Characterization of Pgrmcs and mPRs in oocyte maturation and ovulation in zebrafish (*Danio rerio*)

by

Xinjun Wu

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Director of Dissertation: Yong Zhu, Ph.D., Department of Biology

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It is well known that progestins initiate important reproductive processes such as oocyte maturation and ovulation in vertebrates. Progestins exert functions through binding to progestin receptors. At least three families of progestin receptors including nuclear progestin receptor (Pgr), progestin receptor membrane components (Pgrmcs), and membrane progestin receptors (mPRs) may mediate these actions of progestins. However, knockout studies of these purported progestin receptors in oocyte maturation and ovulation are still rare. The main objective of this study is to determine *in vivo* functions of these purported progestin receptors, especially in oocyte maturation and ovulation *via* individual and combined knockouts of these progestin receptors. We found involvements of Pgr, Pgrmcs, and mPRs in oocyte maturation and oocyte ovulation in zebrafish.

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Xinjun Wu

APPROVED BY:

DIRECTOR OF DISSERTATION: _____
Yong Zhu, Ph.D.

COMMITTEE MEMBER: _____
Peter Thomas, Ph.D.

COMMITTEE MEMBER: _____
Elizabeth Ables, Ph.D.

COMMITTEE MEMBER: _____
Myon Hee Lee, Ph.D.

COMMITTEE MEMBER: _____
Fadi Aziz Issa, Ph.D.

ACTING CHAIR OF THE
DEPARTMENT OF BIOLOGY: _____
David Chalcraft, Ph.D.

DEAN OF THE
GRADUATE SCHOOL: _____
Paul Gemperline, Ph.D.

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CHAPTER 1: Introduction

Chapter summary

Progestins are a group of steroid hormones including progesterone (P4) and its derivatives such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Progestins are important steroid hormones with multiple reproductive functions in vertebrates by acting through multiple progestin receptors. In addition to a well-characterized classical and nuclear progestin receptor (Pgr), two structurally unrelated membrane progestin receptor families, progestin receptor membrane components (Pgrmc1 & 2) and membrane progestin receptors (mPRα, β, γ, δ, ε), have also been proposed. Progestins can signal through their nuclear receptor that acts as a transcription factor (genomic progestin signaling), or rapidly through their cognate receptors at the plasma membrane (nongenomic progestin signaling). It is well established that progestins are essential for genomic action like ovulation and also rapid nongenomic action like oocyte maturation. The receptors responsible for these nongenomic actions of progestin are still being actively sought out. Pgr is a transcription factor that is essential for oocyte ovulation across different species. In addition, Pgr may mediate rapid nongenomic signaling of progestin in oocyte maturation. Pgrmcs have a single transmembrane domain with putative Src homology domains located both on membrane and nucleus. The mPRs have seven integral transmembrane domains and mediate progestin signaling *via* G-protein coupled pathways. Studies conducted so far were limited to a few members of these membrane progestin receptors (e.g. Pgrmc1, mPRα, mPRβ), mainly using overexpression and pharmaceutical approaches in different cell lines and animal models. Evidence using knockouts is still rare for *in vivo* functions and signaling of these membrane-located progestin receptors. Here, I will summarize recent findings on these three families of progestin receptors, emphasizing the findings in oocyte maturation and ovulation.

Introduction

Progestins are a group of steroid hormones including progesterone (P4) and its derivatives such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Progestins are important steroid hormones with multiple reproductive functions in vertebrates. For example, progesterone is essential for oocyte ovulation in mice (Robker et al., 2000). In teleosts, DHP is the maturation-inducing steroid (MIS) in zebrafish and other freshwater fish (Nagahama and Yamashita, 2008; Thomas, 2012). Progestins exert functions through two distinct signaling pathways, i.e., genomic or nongenomic signaling pathways (Thomas, 2003). In addition to the well-established classical genomic signaling pathway progestin acting through nuclear progestin receptor (Pgr), the nongenomic signaling pathway has also been well recognized (Thomas, 2003, 2012; Zhu et al., 2008). The numerous examples in which steroids affect cellular dynamics within seconds to minutes of exposure have remained difficult to explain using the classical genomic signaling mechanism of steroids. Unlike the classical (and slow) steroid signaling pathway, rapid steroid signaling acts *via* receptors located either at the cell plasma membrane or in the cytosol act independently of transcription, and thus are characterized as nongenomic steroid signaling. Nongenomic steroid action involves intracellular secondary messengers and rapid signal-transduction cascades such as ion fluxes (often calcium), cAMP modulation and activation/deactivation of protein kinase pathways (Lösel and Wehling, 2003). However, the identifications of receptors for nongenomic progestin signaling remains controversial.

Genomic signaling pathway of progestin and classical nuclear progestin receptor (Pgr)

In the classical theory, steroids enter cells passively by diffusion through lipid membranes and then are bound to a 'classical' steroid receptor located in the cytosol or nucleus. Ligand binding induces a conformational change in the receptor protein, which is accompanied by the dissociation

of accessory proteins, thereby exposing the DNA-binding domain (Lösel and Wehling, 2003). In the nucleus, the receptor-ligand complex then binds to DNA, leading to changes in transcription. This genomic mechanism of steroid signaling is a relatively slow process that may take hours to days to elicit a biological response because of the required time for transcription and translation (Lösel and Wehling, 2003).

Classically, progestins exert their genomic functions through nuclear progestin receptor (Pgr). PGR was originally identified and characterized in the rodent uterus with two isoforms, named PGR-A and PGR-B (Milgrom et al., 1970). Both PGR isoforms have a similarly high affinity for progesterone at a low nanomolar range of capacity (Lessey et al., 1983). In zebrafish, only a single locus encoding one full-length Pgr has been reported (Diotel et al., 2011; Hanna et al., 2010). Pgr exerts its functions as a ligand-activated transcription factor (Mulac-Jericevic and Conneely, 2004).

Nongenomic signaling pathway of progestin and its receptors

Nongenomic signaling of progestins are well-known. Physiological processes regulated by nongenomic progestin signaling include rapid changes of sexual behavior, induction of acrosomal reaction in sperm, rapid increase in sperm motility, modulation of GnRH discharge in brain, regulation of ion flux in neurons, activation of protein kinase C in the hypothalamus, vascular smooth muscle and epithelial cells, rapid activation of second signal pathways in breast cancer, the influx of calcium in hepatocytes, and resumption of oocyte maturation in fish and amphibian species (Baldi et al., 1998; Blackmore et al., 1990; Calogero et al., 1998; Faivre et al., 2005; Ferrell Jr, 1999; Foresta et al., 1993; Frye et al., 2006; Kostellow et al., 1980; Luconi et al., 2004; Majewska et al., 1986; Sabeur et al., 1996; Sim et al., 2001; Thomas et al., 2002). Interestingly, some of these physiological processes still occur despite the absence of Pgr (Bar et al., 2000;

Ehring et al., 1998; Frye et al., 2006; Matsumoto et al., 2002; Sleiter et al., 2009). In addition to classical Pgr, two distinct families of membrane proteins, progestin receptor membrane components (Pgrmcs) and seven transmembrane membrane progestin receptors (mPRs) have been suggested to mediate nongenomic progestin signaling (Thomas, 2008; Zhu et al., 2008).

Progestin receptor membrane components (Pgrmcs)

Pgrmc1 and its paralog, Pgrmc2, belong to the membrane-associated progestin receptor (MAPR) protein family that are found in all eukaryotes, and are mutlifunctioban proteins (Cahil, 2007; Losel et al., 2008; Ryu et al., 2017) with an extraordinary diverse array of proposed funtions, including serving as progestin receptors (Falkenstein et al., 1996; Meyer et al., 1996; Rohe et al., 2009). Pgrmc1 contains the functional domain cytochrome b5-like heme/steroid-binding domain enabling Pgrmc1 to bind to enzymes involved in steroid hormone synthesis and therefore enhance the activity of these enzymes (Cahill, 2007; Mifsud and Bateman, 2002; Pedram et al., 2007). For example, it has been demonstrated that Pgrmc1 binds to Cyp51A1 and is required for its activity in cholesterol synthesis (Kabe et al., 2016; Pedram et al., 2007). Pgrmcs also have several potential Src homology domains for potential interactions with signaling molecules (Peluso et al., 2007).

So far, only two studies showed limited steroid binding characteristics of PGRMC1 (Peluso et al., 2008b; Meyer et al., 1996). Peluso showed that P4 binds competitively and reversibly to PGRMC1 (K_d= 35nM). Recombinant expression of Pgrmc1 increases P4 binding in immortalized granulosa cells (Peluso et al., 2008b). PGRMC1 isolated and purified from porcine liver also seem to bind P4 (apparent K_d of 11nM) (Meyer et al., 1996). However, bacterially expressed PGRMC1 fails to bind P4 (Ghosh et al., 2005; Min et al., 2005), but this may be caused by improper folding or the absence of post-translantional modification, which are characteristic of the bacterially expressed protein(Peluso et al., 2008b). Pgrmc1 also can bind to a variety of ligands, including

heme, cholesterol, and androgens, glucocorticoids (Cahill, 2007; Menzies et al., 1999; Min et al., 2004; Thomas, 2008). Pgrmc1 is also regulated by P4 and takes part in P4 metabolism, which is tissue-specific (Rohe et al., 2009). Pgrmc1 has been suggested to mediate the actions of P4 to activate protein kinase G, MAPK or other rapid signaling mechanisms in mammalian cells, however direct evidence for this is lacking (Lösel et al., 2008). Pgrmc1 may also mediate antiapoptotic and antimitotic actions of P4 in granulosa cells, as well as in ovarian and endometrial cancer cell lines (Peluso et al., 2006). An antiserum against Pgrmc1 attenuated the induction of acrosomal reaction by P4 in human spermatozoa (Lösel et al., 2008). Additionally, Pgrmc1 is involved in axonal guidance during embryogenesis, cell mitosis, cholesterol regulation, polycystic ovarian syndrome or premature ovarian failure, alteration of reproductive behaviors, endocytosis, a component of a P4 receptor with plasminogen activator inhibitor RNA binding protein-1 (PAIRBP-1), and an adapter protein (Cahill, 2007; Peluso et al., 2006; Thomas, 2008; Thomas et al., 2014). However, Thomas et al. also proposed Pgrmc1 might only act as an adaptor protein, and he suggested that previously ascribed progestin functions to Pgrmc1 may dependent on cell surface expression of mPRα (Guo et al., 2016; Thomas et al., 2014). In contrast, only limited studies have been conducted on the closely related Pgrmc2 (Clark et al., 2016; Pru and Clark, 2013).

Conditional ablation of Pgrmc1 results in subfertility in female mice, and knocking out Pgrmc2 causes premature reproductive senescence in females (Clark et al., 2016; McCallum et al., 2016). However, the underlying molecular mechanisms for Pgrmcs (Pgrmc1 and Pgrmc2) signaling and whether Pgrmcs knockout will affect reproduction in other species are still unclear. Pgrmc1 is detected in the inner zones of the rat adrenal cortex, suggesting roles related to steroid synthesis or metal metabolism (Raza et al., 2001). Higher progesterone levels were found in

Pgrmc2$^{-/-}$ but not *Pgrmc1*$^{-/-}$ mice (Clark et al., 2016; McCallum et al., 2016). But how Pgrmc2 affects P4 and other steroid metabolism *in vivo* is still unknown.

Membrane progestin receptors (mPRs)

At the beginning of this century, a group of G protein-coupled receptor (GPCR) like proteins that have a high affinity for progestin binding, named membrane progestin receptors (mPRs), were identified and characterized in fish, frogs, and mammals (Zhu et al., 2003a; Zhu et al., 2003b). This mPRs belong to the progestin and adipoQ receptor superfamily (PAQR) (Thomas, 2012; Thomas et al., 2007). The mPRs have GPCR-like structures including seven transmembrane domains, N-terminus on the outside of the cell and a C-terminus on the inside of the cells, coupling to heterotrimeric G proteins, and mediation of rapid nongenomic signaling (Thomas et al., 2007). But mPRs and GPCR are evolved from different origins. Phylogenetic analysis indicates mPRs and their PAQR receptors are descendants of hemolysins, which are restricted to *Eubacteria*; while GPCRs are direct descendants of bacterial rhodopsin, originating in *Archaebacteria* (Thomas et al., 2007). The mPRs have established association with G_i inhibitory proteins, but the exact nature of the G protein/receptor interactions are unknown since they are not typical GPCRs (Thomas et al., 2007).

Five mPR subtypes (mPRα, mPRβ, mPRγ, mPRδ, and mPRε) are found in all vertebrates. Zebrafish has two additional mPR paralogs, likely due to teleost specific genome duplication (Glasauer and Neuhauss, 2014). mPRα mediates rapid actions of progestins in every cell type that non-classical progestin actions have been described. In fully grown oocytes, mPRα is critical for progestin-induced final oocyte maturation (Hanna and Zhu, 2011; Qiu et al., 2008; Thomas, 2003). mPRs are most likely to be the receptors that mediate progestin signaling that inhibit intracellular cAMP formation, and therefore inhibit GnRH release in Pgr-KO mice (Sleiter et al., 2009).

Experimental evidence supports that mPRα is intermediary in progestin-induced cell survival (Thomas, 2008). mPRs also contribute to sperm hyper-motility (Falkenstein et al., 1999; Thomas, 2003) and involvement in the female menstrual cycle and labor induction (Fernandes et al., 2005). The roles of the different mPRs in reproduction are emerging areas of research and the evidence of mPRs' functions, especially *in vivo* in knockouts, are still lacking.

Potential interactions between different progestin receptors

The precise function and requirements of each of these progestin receptors in the wide array of nongenomic progestin actions that have been identified to date are unclear. The interactions between different progestin receptor families also make progestin signaling more complicated. Genomic and nongenomic actions of progestin signaling are not exclusive to each other. Activation of membrane-localized receptors can (and often do) ultimately lead to activation of nuclear response elements and transcriptional changes. For example, rapid progestin activation of signaling pathways may play a role in gene regulation by mediating phosphorylation of Pgr and/or Pgr associated coregulatory proteins. Several phosphorylation sites in Pgr are rapidly increased by P4 treatment and have been implicated in controlling the transcriptional activity of Pgr (Weigel, 1996; Weigel and Moore, 2007).

Pgr also can coordinate gene expression by indirect mechanisms involving interaction with SRC tyrosine kinase and subsequent activation of the MAPK pathway. Since MAPK is capable of phosphorylating and activating nuclear transcription factors, this raised the possibility that Pgr activation of Src/MARK pathway may provide alternative means for P4 regulation of gene transcription, independent of the direct nuclear transcription activity of Pgr (Boonyaratanakornkit et al., 2008). Also, P4 may mediate gene expression independent of Pgr. Indian hedgehog (Ihh), for example, a gene essential for uterine function and fertility in mice, is transiently upregulated

in $PGR^{-/-}$ female mice in response to P4 treatment *in vivo* (Lee et al., 2006). A few additional examples showing the interactions between progestin receptors are described below.

Possible compensatory role between Pgr and Pgrmc1

Using in situ proximity ligation assays (PLAs), a close interaction between Pgr and Pgrmc1 is observed mainly in the cytoplasm and to a lesser extent in the nucleus of many granulosa/luteal cells (Sueldo et al., 2015). In addition, the Pgrmc1 levels in the brains of Pgr-KO female mice were higher than wild-type littermates (Krebs et al., 2000). Activation of Pgr also represses expression of Pgrmc1 during lordosis facilitation (Krebs et al., 2000). These might indicate a compensatory regulatory mechanism between Pgr and Pgrmc1 (Krebs et al., 2000).

Co-localization of Pgrmc1 and mPRs

Localization of Pgrmc1 and mPRα are quite close. Immunocytochemical and coimmunoprecipitation studies showed a close association of Pgrmc1 with mPRα in spontaneously immortalized rat granulosa cell membranes (Thomas et al., 2014). Overexpression of Pgrmc1 increased expression of mPRα on cell membranes that are associated with increased specific P4 binding (Thomas et al., 2014). There are no reports of G protein activation through Pgrmc1. Therefore, Thomas et al. proposed Pgrmc1 might act as an adaptor protein, transporting mPRα to the cell surface (Thomas et al., 2014). Previously ascribed progestin functions to Pgrmc1 may dependent on cell surface expression of mPRα (Guo et al., 2016; Thomas et al., 2014).

Activation of mPRs increases Pgr expression

Co-localization, interaction, and crosstalk in signaling between mPRs and classical Pgr have been demonstrated in human myometrial cells (Karteris et al., 2006). Activation of mPRs signaling *via* Gi proteins led to the transactivation of PGR-B in human myometrial cells (Karteris et al., 2006). mPRα has been shown to influence PGR transactivation by downregulating steroid

receptor coactivator 2 (SRC2) in human myometrial cells collected at the end of pregnancy (Karteris et al., 2006). In addition, a significant decrease of Pgr mediated reporter activity was observed in human myometrial cells cotransfected with mPRα and β siRNA (Karteris et al., 2006).

Oocyte maturation

Oocyte maturation is a resumption of meiotic arrest from prophase I to metaphase II of meiosis. Oocyte maturation occurs before ovulation and is a prerequisite for successful fertilization. Progestins play essential roles in oocyte maturation in fish and frogs (Kalinowski et al., 2004; Pace and Thomas, 2005). In contrast, the role of progestins in oocyte maturation in mammals is still controversial (Downs et al., 1989; Eppig, 1991; Eppig and Downs, 1987; Shim et al., 1992). In all animals, oocytes that have completed the growth phase (post-vitellogenic oocytes in non-mammalian models) cannot be fertilized because the first meiotic division is incomplete. Most of the arrest is due to high levels of intracellular cAMP (Duckworth et al., 2002). The prerequisite for resumption of meiosis and oocyte maturation is a decrease of cAMP levels in oocytes. A decrease in cAMP is mediated through activation of pertussis toxin-sensitive inhibitory G protein (Gi) pathway in fish (Pace and Thomas, 2005) but *via* a stimulatory G-protein (Gs) pathway in frogs (Kalinowski et al., 2004). Both pathways, in fish and frogs, are triggered by progestin signaling. Specific progestins can cause the resumption of final oocyte maturation in amphibians and fish species. For example, progesterone (P4) is the inducer of meiosis in amphibian oocytes (Masui, 1967); 4-pregnen-17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) in perciform fish such as the spotted seatrout (Pinter and Thomas, 1999; Trant and Thomas, 1989); and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) in salmon (Nagahama and Adachi, 1985) and zebrafish (Van den Hurk et al., 1987).

A dramatic shift in the pathway of steroid syntheses is also required for oocytes to initiate the maturation process. Plasma levels of progestins are increased before maturation and ovulation as the estradiol level is decreased (Andersen, 1990; Mendoza et al., 2002). With this dramatic increase in progestins, membrane progestin receptors (mPRα and mPRβ) also are up-regulated in fully-grown immature oocytes prior to oocyte maturation (Hanna and Zhu, 2011). Progestins bind to their membrane located receptors to decrease cAMP levels and resume meiosis, promoting germinal vesicle breakdown (GVBD), chromosome condensation, and formation of the first polar body (Nagahama and Yamashita, 2008; Thomas, 2012; Zhu et al., 2008). Previous studies indicate all three progestin receptor families are involved in oocyte maturation in different ways (Aizen et al., 2018; Aizen and Thomas, 2015; Bayaa et al., 2000; Hanna and Zhu, 2011; Tian et al., 2000).

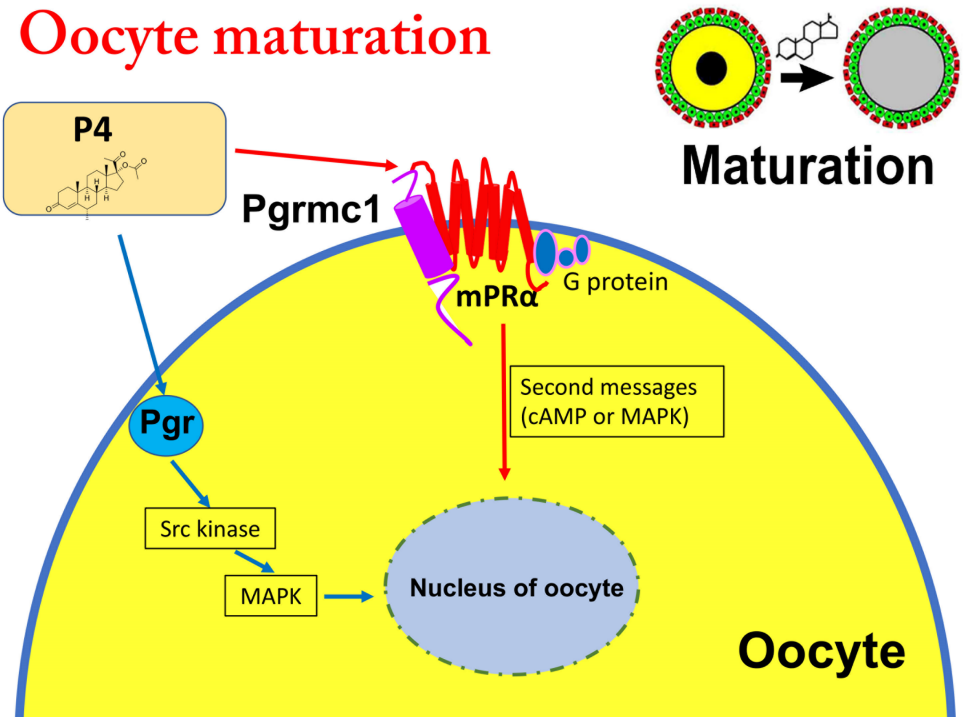


Figure 1. 1 Different families of progestin receptors in oocyte maturation. A small fraction of Pgr can be detected on the oocyte membrane, which can bind with Src kinase and then activate MAPK signal pathway. Activated MAPK then induces oocyte maturation. mPRα functions as plasma membrane-bound receptor coupled to G protein mediating rapid actions of progestin *via* activation of an inhibitory G protein (Gi) and suppression of adenylyl cyclase activity and cAMP

production. Pgrmc1 likely facilitates oocyte maturation through regulating oocyte plasma membrane localization of mPRα and regulate mPRα activity.

The role of Pgr in oocyte maturation remains unclear

Pgr contains a nine amino acid motif that mediates palmitoylation-induced membrane translocation and facilitates ~5% Pgr located on the membrane (Pedram et al., 2007). PGR-B is naturally distributed between cytoplasm and nucleus mediated progestin activation of Src/MARK, whereas PGR-A is predominantly nuclear (Boonyaratanakornkit et al., 2008). This membrane located Pgr may play a role in oocyte maturation. Selective nuclear P4 receptor agonist, R5020, did not cause G protein activation (Dressing et al., 2010). Instead, human PGR contains a polyproline motif in the amino-terminal domain (aa 421-428) that interacts with the SH3 domain of Src and mediates rapid activation of c-Src and downstream MAPK (Boonyaratanakornkit et al., 2008). Pgr also acts *via* rapid, extra-nuclear, signaling cascades, such as PI3K/ Akt (Boonyaratanakornkit et al., 2008).

Pgr mediates rapid nongenomic progestin signaling in several cell types, including immortal breast cancers and amphibian oocytes (Bayaa et al., 2000; Faivre et al., 2005; Tian et al., 2000). Two nuclear progestin receptors (XPR-1 and XPR-2) have been identified in *Xenopus* (Bayaa et al., 2000; Tian et al., 2000). Over-expression of XPR-1 or XPR-2 accelerates progesterone-induced oocyte maturation and cell cycle re-entry, while XPR-1 antisense inhibits the process (Bayaa et al., 2000; Tian et al., 2000). Over-expression of Pgr increases rapid intracellular nongenomic signaling in response to progestins resulting in activation of MAPK and cell cycle regulators such as cyclins in both *Xenopus* oocytes and human breast cancer cells (Bayaa et al., 2000; Boonyaratanakornkit et al., 2007; Faivre et al., 2005; Skildum et al., 2005; Tian et al., 2000). Similarly, microinjection of Pgr transcripts into immature oocytes accelerated oocyte maturation in zebrafish oocytes (Hanna and Zhu, 2011). However, unlike the nongenomic pathway,

the acceleration of maturation in the Pgr injected group was blocked by treatment with transcription inhibitor actinomycin D, implying a requirement of a genomic signaling pathway (Hanna and Zhu, 2011).

The signaling pathway of Pgr mediated nongenomic progestin signaling remains unclear. Human PGR-B has a proline-rich domain within its amino terminus, which could interact with SH3 domain of Src upon binding of progesterone, thus activating downstream MAPK signaling in human breast cancer cells (Boonyaratanakornkit et al., 2001). However, this amino-terminal proline-rich motif is not conserved in several other model organisms such as mice, *Xenopus* or zebrafish (Hanna, 2009). Knockout of Pgr in zebrafish clearly indicates that Pgr is not essential for oocyte maturation in zebrafish (Zhu et al., 2015).

Pgrmc1 acts as an adaptor in oocyte maturation while the roles of Pgrmc2 are unknown

Using biochemical and pharmacological approaches, a decrease in oocyte maturation in response to DHP was obtained when a Pgrmc1 inhibitor, AG205, was used in an *in vitro* zebrafish oocyte maturation assay (Aizen et al., 2018; Aizen and Thomas, 2015). Pgrmc1 antibody injection also significantly lowered the percentage of bovine oocytes that matured and reached the metaphase II stage after 24 hours of culture (Luciano et al., 2010). Pgrmc1 is unlikely to directly regulate oocyte maturation, as there is no evidence for a direct interaction between Pgrmc1 and the inhibitory G-protein known to be important for meiosis resumption in fishes. Pgrmc1 likely facilitates oocyte maturation through regulating oocyte plasma membrane localization of mPRα (Aizen et al., 2018). Pgrmc1 co-immunoprecipitates with mPRα in breast cancers (Thomas et al., 2014) and manipulation of Pgrmc1 expression with siRNA transfection and AG205 decreases plasma membrane localization of mPRα in zebrafish oocytes resulting in a decrease in oocyte maturation (Aizen et al., 2018). This study also indicates a close association between Pgrmc1 and

mPRα, and between Pgrmc1 and receptor tyrosine-protein kinase erbB-2 (Erbb2) in zebrafish oocytes at the plasma membrane of stage IV follicles using in situ proximity ligation assays (Aizen et al., 2018).

On the other hand, Pgrmc1 was also suggested to be involved in estrogen maintenance of zebrafish oocyte meiotic arrest, *via* regulating G protein-coupled estrogen receptor 1 (Gper)-dependent epidermal growth factor receptor (Egfr) signaling (Aizen and Thomas, 2015). Pgrmc1 regulates the expression of Egfr on zebrafish oocyte membranes and influences the inhibitory effects of estrogens on the resumption of meiosis through Gper. The Pgrmc1 inhibitor, AG205, blocks the stimulatory effect of an Egfr inhibitor in oocyte maturation and decreases expression of Egfr on the oocyte membrane (Aizen and Thomas, 2015). Therefore, Pgrmc1 may inhibit or stimulate oocyte maturation by acting as an adaptor protein involved in membrane trafficking of mPRα, Egfr, Erbb2, or Gper depending on the development stage of the oocytes and/or serum concentrations of estrogens and P4.

During bovine oocyte maturation, the location of Pgrmc1 changes dramatically. With the resumption of meiosis *in vitro*, Pgrmc1 concentrated at the centromeric region of metaphase I chromosomes, while in the anaphase I/telophase I stage the majority of Pgrmc1 concentrated between the separating chromosomes. At the metaphase II stage, Pgrmc1 re-associated with the centromeric region of the chromosomes (Luciano et al., 2010). In addition, a colocalization study demonstrated that Pgrmc1 associated with the phosphorylated form of aurora kinase B, which localizes to the centromeres at metaphase (Luciano et al., 2010; Terzaghi et al., 2016). These suggest Pgrmc1 may play a role in bovine oocyte maturation directly.

In contrast, studies about Pgrmc2 are rare and the roles of Pgrmc2 in oocyte maturation remain unclear.

mPRs play a direct role in oocyte maturation

mPRα appears to function as plasma membrane-bound receptor couple to G protein mediating rapid actions of progesterone *via* activation of an inhibitory G protein (Gi) and suppression of adenylyl cyclase activity and cAMP production (Thomas, 2008; Zhu et al., 2003a). The protein of mPRα is localized on the plasma membranes of both granulosa cell and theca cells, though some of the receptors appeared to be trapped within the intracellular membrane compartments of Atlantic croaker and zebrafish ovaries (Dressing et al., 2010; Hanna and Zhu, 2011). The mPRβ expression was rather variable, localizing to the intracellular membrane or cytoplasmic areas within cells (Hanna and Zhu, 2011). The expressions of mPRα and mPRβ transcripts and proteins were abundant and increased significantly in late-stage denuded oocytes prior to oocyte maturation and can be induced by DHP in follicle-enclosed oocytes (Hanna and Zhu, 2011). The expression of mPRα is also induced by gonadotropins during seatrout oocyte maturation followed by a dramatic decrease in ovulated oocytes (Zhu et al., 2003b). Over-expression of mPRα also increases cyclin B production in follicle-enclosed oocytes concomitantly with the increase in maturation while over-expression of mPRβ has no such effect (Hanna and Zhu, 2011). Antisense microinjection of mPRα and (or) mPRβ can block MIS-induced oocyte maturation (Thomas et al., 2004; Tokumoto et al., 2006; Zhu et al., 2003a).

In human myometrial cells, both mPRα and mPRβ are involved in the downregulation of adenylyl cyclase activity through a pertussis toxin-sensitive Gi pathway (Karteris et al., 2006). Thomas et al. also reported that mPRα and mPRβ function similar to GPCRs because progesterone activates a pertussis toxin-sensitive G(i) pathway to down-regulate membrane-bound adenylyl cyclase activity in mPRα-transfected cells (Thomas et al., 2007). Signaling of the zebrafish mPRα was blocked by pertussis toxin, implying activation of a Gi protein, while sensitivity to pertussis

or cholera toxin was not shown with mPRβ-mediated signaling, possibly indicating that this receptor activates a different pertussis toxin-insensitive G protein in nuclear progesterone receptor-deficient human breast carcinoma lines (Hanna et al., 2006). Over-expression of mPRα in follicle-enclosed oocytes significantly increased the activity of MAPK, the production of cyclin B protein, and the number of oocytes that underwent oocyte maturation without exogenous progestin, while over-expression of mPRβ alone had no such effect. Therefore, mPRα, but not mPRβ, conveys extracellular non-genomic progestin signaling to initiate meiosis resumption in follicle-enclosed zebrafish oocytes (Hanna and Zhu, 2011). Interestingly, mPRβ appears to be the major membrane progestin receptor responsible for oocyte maturation in *Xenopus*, since mPRα appears to be absent in this species (Josefsberg Ben-Yehoshua et al., 2007).

Oocyte ovulation

In addition to oocyte maturation, progestins also are essential for ovulation. Ovulation means follicle rupture and release of the mature oocyte from the ovary. A preovulatory surge of luteinizing hormone (LH) is essential for ovulation in all vertebrates (Stagey et al., 1979). LH secreted from pituitary binds to its receptor (Lhcgr) at granulosa cells to initiate a cascade of signaling, eventually leading to follicular rupture (Chu et al., 2014; Takahashi et al., 2016; Zhang et al., 2015). Under the control of LH, several genes are induced in ovarian follicular cells which are critical for ovulation, including Pgr. In follicular cells, several proteins essential for steroidogenesis are induced in the ovary during gonadotropin-induced ovulation, including steroidogenic acute regulatory protein (Star) and cholesterol side-chain cleavage enzyme (Cyp11a1; P450scc) (Ronen-Fuhrmann et al., 1998). Progestin signaling has been shown to be critical in ovulation (Tanaka et al., 1991).

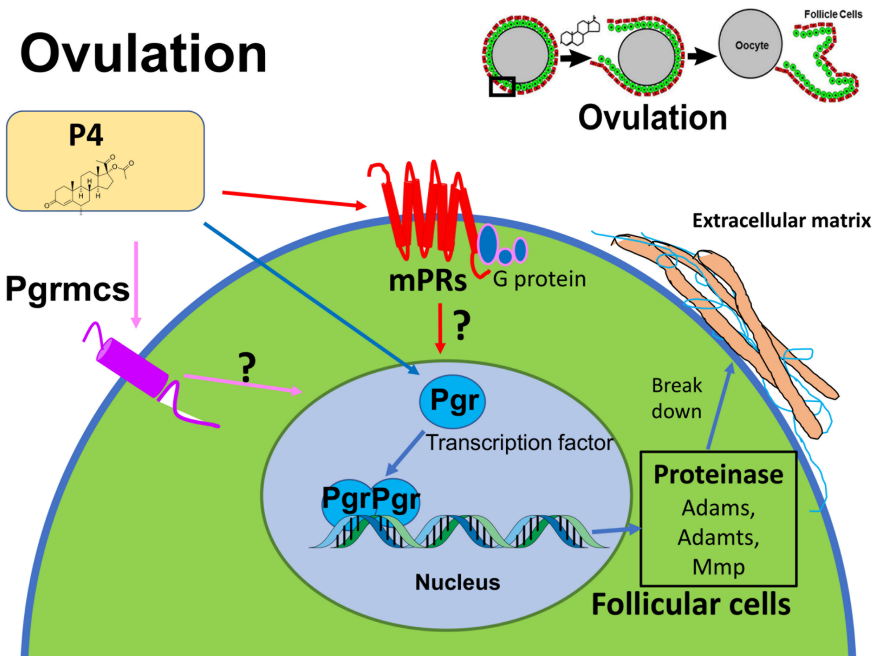


Figure 1. 2 Three different progestin receptor families in oocyte ovulation. To successfully ovulate, folliclar cells need to synthesis proteinases to breakdown the extracellular matrix. These proteinases include Adams (a disintegrin and metalloprotease), Adamts (a disintegrin and metalloproteinase with thrombospondin motifs) and Mmp (matrix metallopeptidases) families. Progestin is essential for ovulation signaling through Pgr. Pgr is a transcription factor regulating the expression of proteinases that can breakdown extracellular matrix and release the oocyte. However, the functions of Pgrmcs and mPRs in the folliclar cells remain unknown.

Functions of Pgr in ovulation are conserved in vertebrates. In Pgr knockout zebrafish, mice, and rats, oocytes were trapped within follicular cells and unable to ovulate, leading to infertility (Kubota et al., 2016; Lydon et al., 1995; Zhu et al., 2015). Blockade of Pgr function with a selective antagonist RU486 or CDB-2914 also reduced the number of ovulated oocytes in mice (Loutradis et al., 1991; Palanisamy et al., 2006). A few genome-wide transcriptome analyses in humans, macaques, mice, and zebrafish suggest conserved gene expression and signaling pathways in the follicular cells of preovulatory oocytes (Hernandez-Gonzalez et al., 2006; Liu et al., 2017; Wissing et al., 2014; Xu et al., 2010). Liu et al. identified ovulatory pathways and processes such as inflammatory response, angiogenesis, cytokine production, cell migration, chemotaxis, MAPK,

focal adhesion, and cytoskeleton reorganization are downregulated in the follicular cells of Pgr KO female zebrafish, which are unable to ovulate (Liu et al., 2017). In contrast, genes involved in DNA replication, DNA repair, DNA methylation, RNA processing, telomere maintenance, spindle assembling, nuclear acid transport, catabolic processes, and nuclear and cell division are upregulated in the follicular cells of Pgr KO zebrafish (Liu et al., 2017).

The essential role of Pgr in oocyte ovulation is well known (Kubota et al., 2016; Lydon et al., 1995; Zhu et al., 2015). Pgrmcs and mPRs also are expressed in the follicular cells, but their roles in oocyte ovulation are still unknown. Previous studies showed Pgrmc1 localizes to the nucleus to regulate gene expression in response to P4 in spontaneously immortalized granulosa cells (Peluso et al., 2012). However, Pgrmc1 does not have a DNA-binding domain; as such, Pgrmc1 may function as a scaffold protein in a transcriptional complex (Peluso et al., 2012). P4 can exert inhibitory effects on gonadotropin-releasing hormone (GnRH) release in the absence of Pgr, which may be mediated by mPRs (Sleiter et al., 2009). By regulating GnRH, mPRs may also regulate oocyte ovulation indirectly.

Significance of studies

Previous studies using overexpression or knockdown suggest that all three families of progestin receptors mediate progestin signaling to induce oocyte maturation (Bayaa et al., 2000; Tian et al., 2000; Zhu et al., 2003b). However, knockout studies of these progestin receptors in oocyte maturation are still lacking. It is well established that progestin and Pgr are essential for ovulation in vertebrates. However, whether Pgrmcs and mPRs also mediate progestin signaling in ovulation is unknown, as both families of the progestin receptor are expressed in follicular cells. A better understanding of these progestin receptors regulated physiological processes including

oocyte maturation and ovulation will advance our knowledge and may also facilitate the development of treatments for infertility or fertility control. The generation of single, double, and higher-order knockout lines that cover the known spectrum of progestin receptors is expected to resolve the debate concerning the basic functions of these receptors, and reveal their roles in slow-response genomic and rapid-response nongenomic progestin signaling.

CHAPTER 2: Pgrmc1 Knockout in Zebrafish Impairs Oocyte Maturation Which Involves Reduced Expression of mPRα (Paqr7)

Wu, X.J., Thomas, P., Zhu, Y., 2018. Pgrmc1 Knockout Impairs Oocyte Maturation in Zebrafish. Frontiers in Endocrinology 9, 560.

Chapter summary

Recent investigations suggest progestin receptor membrane component 1 (PGRMC1) associates with and transports a wide range of molecules such as heme, cytochromes P450, steroids with 21 carbons, membrane progestin receptor alpha (mPRα/Paqr7), epidermal growth factor receptor, and insulin receptor. It is difficult to differentiate the true functions of PGRMC1 from the functions of its associated molecules using biochemical and pharmacological approaches. To determine the physiological function(s) of PGRMC1, global knockouts for *pgrmc1* (*pgrmc1*$^{-/-}$) in zebrafish were generated. A reduction in both spawning frequency and the number of embryos produced by female mutants were found. Fully-grown immature oocytes also showed reduced sensitivity to a progestin hormone and a reduced number of oocytes undergoing meiotic maturation both *in vivo* and *in vitro* in *pgrmc1*$^{-/-}$. This reduced sensitivity to progestin corresponds well with significant reduced expression of mPRα, the receptor mainly responsible for mediating oocyte maturation and meiosis resumption in fish. The results provide *in vivo* and *in vitro* evidence for the physiological functions of Pgrmc1 in oocyte maturation and fertility, as well as a plausible molecular mechanism *via* regulation of mPRα, which in turn directly regulates oocyte maturation and affects fertility in zebrafish.

Introduction

Progestin membrane receptor component 1 (PGRMC1) is a single transmembrane heme-binding protein containing a cytochrome b_5 (Cytb5) motif (Ghosh et al., 2005; Meyer et al., 1996), which has been proposed as a progesterone binding protein in vertebrates (Falkenstein et al., 1996; Meyer et al., 1996; Peluso and Pru, 2014). Various molecules such as heme, fatty acid 2-hydroxylase, cytochrome P-450 enzymes, insulin receptor, epidermal growth factor receptor (EGFR), Erbb2, and membrane progestin receptor α (mPRα, PAQR7) can interact and associate with Pgrmc1 (Ahmed et al., 2010; Aizen et al., 2018; Hampton et al., 2018; Hughes et al., 2007; Kabe et al., 2016). Elucidating the roles of PGRMC1 has been further complicated because of its broad localization in various organelles and its purported role in transporting other molecules (Ryu et al., 2017; Thomas, 2017). Untangling the roles of PGRMC1 from functions of its associated molecules requires further studies such as generating and characterizing PGRMC1 knockouts. Conditional knockout of PGRMC1 results have suggested subfertility in female mice (McCallum et al., 2016). However, conditional ablation of PGRMC1 using promoter of Amhr2 (cre) mice to delete PGRMC1 resulted in the same number of pups/litter as wildtype (*wt*) mice, although conditional ablation using the promoter of nuclear progesterone receptor, Pgr (cre) caused a 40% reduction in the number of pups/litter. It is still unclear whether PGRMC1 affects female fertility in non-mammalian vertebrates that lack a uterus, and whether the difference in fertility observed between Amhr2- and Pgr- induced cre knockouts was due to off-target effects or inefficiency of *LoxP* site recombination frequently found in conditional knockouts.

To address these questions, we generated Pgrmc1 mutants in zebrafish (*Danio rerio*) using CRISPR/Cas9 gene editing technology, and characterized the phenotypes of the knockouts, and also examined the molecular mechanisms underlying the actions of Pgrmc1. Reductions in the

spawning frequencies and the number of embryos produced were found in Pgrmc1 mutant females. Expression of mPRα was significantly reduced in the Pgrmc1 knockouts, which is likely the cause, at least partially, of the attenuation of oocyte maturation in Pgrmc1 knockouts. Unless indicated otherwise, we will use P4 (progesterone), DHP (17α,20β-dihydroxy-4-pregnen-3-one) or progestin interchangeably in this manuscript because of conserved physiological functions and shared structure of these progestins.

Materials and methods

Animals

The zebrafish (*Danio rerio*) strain used in this investigation, the Tübingen strain, was initially obtained from the Zebrafish International Resource Center, and then propagated in our laboratory at East Carolina University following previously published guidelines (Zhu et al., 2015). All the animal care and use protocols were approved by Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

Design of Pgrmc1 knockout targets and preparation of Cas9 RNA and sgRNAs

We identified 5'GG-(N_{18})-NGG3' target sequences in exon 1 of *pgrmc1* (GGAGACAAGCCTGCAGACTATGG) (Figure 2.1). Syntheses of Cas9 RNA and single guide RNAs (sgRNAs) were based on a protocol described previously (Jao et al., 2013). For synthesis of Cas9 transcripts (nls-zCas9-nls RNA), a template plasmid (pCS2-nls-zCas9-nls) was linearized by NotI digestion, then purified using a QIAprep column (Qiagen, Germantown, MD, USA). Capped Cas9 mRNA (nls-zCas9-nls) was synthesized using mMESSAGE mMACHINE SP6 kit (Fisher Scientific, Hampton, NH, USA) and purified using a RNeasy Mini kit (Qiagen). For guide RNAs (gRNAs), template plasmids were linearized by BamHI digestion and purified using a

QIAprep column. The gRNAs were generated by *in vitro* transcription using a MEGAshortscript T7 kit (Fisher Scientific). The size and quality of the resulting gRNA was confirmed by electrophoresis using a 2% (wt/vol) formaldehyde agarose gel.

Establishment of zebrafish knockout line for Pgrmc1

Screening and generation of mutant zebrafish lines for Pgrmc1 followed protocols established previously (Zhu et al., 2015). To generate a founder population (F0), fertilized eggs were collected within 5 minutes of natural spawning of wildtype fish from their crossing tanks that were set up the night before. Microinjection was performed on newly fertilized zebrafish embryos at one or two-cell stages. Approximately 100 ng/µl of sgRNA and 150 ng/µl of Cas9 mRNA were co-injected into the embryo using a glass microcapillary pipette attached to a micromanipulator under a stereomicroscope (Leica MZ6). Injection was driven by compressed N_2 gas, under the control of a PV820 Pneumatic PicPump (World Precision Instrument, Florida, USA). For comparison, and to estimate mutagenesis efficiency, embryos without microinjection were designated as wildtype and used as controls. A pool of genomic DNA was extracted from 30 normally developing wildtype or CRISPR/Cas9-gRNA-microinjected embryos two-day post fertilization (dpf) using a HotSHOT method (Meeker et al., 2007). Mutation rates were estimated by comparing band intensities of undigested PCR products to intensities of digested PCR products using T7 endonucleases I assay (Zhu et al., 2015). The PCR products were cleaned through a Qiagen column, cloned into a TA cloning vector, and potential mutant clones were selected for DNA sequencing analysis to confirm the presence of frame-shifting mutation (Zhu et al., 2015).

To identify germline-transmitted mutations, remaining F0 founder embryos were raised to adulthood and outcrossed with wildtype fish. Genomic DNA from each cross was extracted from 30 randomly selected and pooled F1 embryos, and the status of the target site was analyzed *via*

PCR amplification, T7 Endonucleases I assay, and DNA sequencing as described above. The remaining F1 embryos with identified frame shift mutations were raised to adulthood and were genotyped individually. Genomic DNA was extracted from part of the caudal fin of adult fish in a 50 µl hot alkaline solution and analyzed as stated above. Heterozygous F1 adults carrying the same frameshift mutant alleles were crossed with each other. These crosses yielded wildtype, heterozygous, and homozygous F2 fish that were further characterized genetically and physiologically. A mutant-specific reverse primer was then designed according to the mutated sequence in CRISPR/Cas9-induced mutation (*Pgrmc1*-Forward: 5'-GGGGGTGTCTGAAAGGAACT-3' *Pgrmc1*-wildtype Reverse: 5'-CTCCTCAACCGGGCCATAGT-3' *Pgrmc1*-mutant Reverse: 5'-GGCTCCTCAACCGAGACAAA-3'). PCR condition was optimized for efficient identification of the specific mutation. After an initial denaturation for 2 minutes at 94 ℃, the cycling reaction was performed with the profile of 30 s at 94 ℃, 30 s at 61 ℃ and 45 s at 72 ℃ for 35 cycles, followed by a 10 minutes extension at 72 ℃ with a Thermal Cycler (Eppendorf, Hauppauge, NY, USA).

Spawning and fertility

After zebrafish reached their maturity at ~ 4 months of age, at least 10 homozygous mutant female fish were crossed with fertility confirmed wildtype males to check fertility of females. Production of offspring for each genotype was recorded daily for a period of two weeks following a two-week acclimation period. Spawning frequency is defined as the number of times a female produce fertilized embryos in a two-week examination period.

Ovarian histology

Three females from each genotype were collected at 8 am (one hour before lights turned on) and deeply anesthetized in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes. To ensure death, the spinal cord and blood supply were cut off behind the gill cover using sharp scissors. Ovaries were quickly removed and examined by histology following protocols described previously (Sullivan-Brown et al., 2011). Briefly, fresh ovaries were fixed overnight in 10% buffered formalin (Fisher Scientific), washed in tap water, dehydrated through increased concentrations of ethanol (70%, 80%, 90%, 100%, 30 minutes each) and embedded in JB-4 resin (Polysciences, Warrington, PA). Sections of 5 μm were cut and stained with hematoxylin and eosin. Routine histological procedures will be used to assess the development, growth, and maturation of ovaries from the zebrafish with different kinds of mutants. Each ovary is evaluated based upon morphology and numbers of perinucleolar, cortical alveolar, vitellogenic oocytes, fully-grown immature, mature, and ovulated oocytes. The follicles at different stages in ovarian tissue will be determined based on their size, vitellogenic state, and maturation stage in the serial tissue sections of each whole ovary.

Follicle isolation and quantification

Oocyte maturation and ovulation in zebrafish typically occurs prior to the onset of (day) light, while spawning occurs within 1 hr following the onset of light. Therefore, adult females (n = 7) from each mutant genotype were euthanized at 09:30 am, thirty minutes after laboratory lights were turned on, by placing each fish in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes, then severing the spinal cord and blood supply using IACUC approved procedures. The ovaries of each fish were then immediately dissected out and rinsed in 60% L-15 media (Sigma-Aldrich, St. Louis, MO, USA) containing 15 mM HEPES (pH=7.2). The term “follicles”

means follicular cells and their enclosed oocytes here. Follicles of various sizes were isolated from the ovaries using fine forceps. The diameter of each follicle was measured under a stereomicroscope (SZX7, Olympus, Japan), and recorded. The developmental stages of follicles were divided into five stages based on morphological criteria and physiological events (Selman et al., 1993; Tyler and Sumpter, 1996) with a slight modification: stage I (<140 μm) and II (140-340 μm) previtellogenic follicles; stage III early vitellogenic follicles (340-690 μm); stage IV late vitellogenic follicles (690-730 μm) that are further divided into IVa and IVb two stages, IVa follicles are maturationally competent fully grown immature follicles (IVa), IVb follicles are mature follicles that have undergone oocyte maturation but have not ovulated (IVb); and stage V ovulated follicles are ovulated eggs with no follicle cells attached (730-750 μm).

***in vitro* oocyte maturation assay**

Zebrafish ovarian follicles were isolated and incubated to determine the sensitivities of follicles to a maturation inducing steroid, DHP (Hanna and Zhu, 2011; Pang and Thomas, 2009). Gravid female zebrafish were euthanized humanely and the ovaries were dissected out and then washed several times in 60% Leibovitz L-15 medium (Sigma-Aldrich). Individual ovarian follicles were carefully separated without damaging the follicular cell layers following previously established protocols (Hanna and Zhu, 2011; Liu et al., 2017). Thirty follicles of the same size (follicular diameter of 550-650 μm) were selected and randomly distributed into a 24-well plate (~30 follicles/1 ml medium/well), and treated with 1 μl DHP dissolved in ethanol. Final concentrations for DHP and ethanol in the incubation medium were 5 nM and 0.1%, respectively. As controls, 1 μl ethanol was added into the control wells containing the same number of follicles (~30 follicles/1 ml medium/well) collected at the same time from the same group of individual fish, and the rates of GVBD were also recorded. Incubation of ovarian follicles was continued for

up to 5 hours, with GVBD being scored each half-hour during the incubation period. All experiments were repeated five times to confirm the results.

Membrane receptor binding assay

Membrane binding of progesterone was conducted as described previously (Hanna et al., 2006) using [1,2,6,7 ^{3}H]-progesterone (85 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA). Plasma membrane fractions of ovaries were obtained following established procedures with few modifications (Patiño and Thomas, 1990; Zhu et al., 2003b). The ovaries from wildtype or Pgrmc1 knockout fish were washed with assay buffer and then sonicated for 15s, followed by a 1000 g centrifugation for 7 min to remove any nuclear and heavy mitochondrial material. The resulting supernatant was centrifuged at 20,000 g for 20 min to obtain the plasma membrane fraction. Progesterone receptor binding in the membrane fractions was measured in a single-point assay as described previously (Thomas et al., 2007). One set of tubes contained radiolabeled progesterone (final concentration: 2.6 nM, total binding), and another set also contained cold progesterone competitor at a 400-fold greater concentration (1 μM) to measure nonspecific binding. After the membrane fractions underwent a 30-min incubation at 4 ^{0}C (160 μg membrane protein), the reaction was stopped by filtration (Whatman GF/B filters, presoaked in assay buffer; Fisher Scientific, Pittsburgh, PA, USA). The filters were washed twice with 25 ml assay buffer and the bound radioactivity was measured by scintillation counting. The results were expressed as DPM specific binding of [^{3}H]-progesterone.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from various tissues using the RNAzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to a modified protocol (Liu et al., 2017), and reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Thermo

Fisher). The amount and purity of the RNA was determined using a Nanodrop 2000 (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using SYBR green with a C1000 Touch Thermal Cycler (Bio-Rad). PCR efficiency calculated from the equation of efficiency (EFF) = 10(−1/slope) − 1 and authentic PCR products were confirmed by analyses of melting curves, gel electrophoresis, and DNA sequencing. PCR data were analyzed using the absolute quantitation method, expressed as copies/µg total RNA, and were determined using Ct values of samples and a standard curve from serial known concentrations of plasmids containing different cDNA fragments of target genes. The full names of the genes and the primers used in this study are listed in Table 2. 1.

Table 2. 1 Sequences of real-time PCR primers used in this study.

Name	**Strand**	**Sequences (5'-3')**	**Product size (bp)**
pgrmc1	F	CAGACTATGGCCCGGTTGAGGAG	281
	R	CTGCATGGCATTGAGATCGG	
pgrmc2	F	ACCAAGGTCTTCGACGTGAC	250
	R	ATGGTTCGTCTCCTGGCTTC	
paqr5a	F	TGCCAAAGACTGCGTGCTAA	281
	R	GGCTGTAGAAGCTGAGTGCT	
paqr5b	F	GCTTGGCTTACCATTGCTTCAC	223
	R	AAGGAATGCAAGCAGCGTATG	
paqr6	F	AAGCTCTGGCCAGTCATTCC	193
	R	CGGGACGCCATGTCTGATAA	

paqr7a	F	GTTGTCTGTCTTGCTTCGGC	243
	R	GTCCAGCGCGTTTTTCTTCA	
paqr7b	F	CGTACTTGTGAGTGCAAGGGT	165
	R	GAAACGAGGGATCTGGCGAA	
paqr8	F	TGCTCAGCGCTGTTACCAAG	372
	R	GCAGCTCGTTGTGACATTGG	
paqr9	F	GTCCCACCAATGAGACTGGG	277
	R	TACGCCAATGAGGAACCCAC	
pgr	F	ACAGACAGCATACACCGC	103
	R	TCCACAGGTCAGAACTCC	
lhcgr	F	CGCTCTGATCAACTGGGACA	218
	R	GGCGCTGTTGGCATAAATCC	
adam8b	F	CCTGGCATCCACAATTGCAC	254
	R	CATTACCACAGACAGGCCCA	

Western blotting

Expression of mPRα, the nuclear progesterone receptor (Pgr), and Pgrmc1 in the fully-grown stage IVa immature follicles were confirmed by Western blot analysis using previously characterized polyclonal zebrafish antibodies (Aizen et al., 2018; Hanna and Zhu, 2009, 2011). In brief, stage IVa follicles were collected from adult zebrafish following protocols described previously (Hanna and Zhu, 2011; Liu et al., 2017). Ten stage IVa follicles collected from freshly sacrificed fish were sonicated in 100 µl of 1x SDS sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol) on ice for about 10 short bursts (Sonic Dismembrator,

Fisher Scientific). Protein samples were then immediately boiled for 10 minutes and stored in at -20 ℃ until analysis. Electrophoresis was conducted with 10 µl of each sample (~one follicle) per lane on an 8% SDS PAGE gel (12% for Pgrmc1), and the resulting protein bands were transferred to a nitrocellulose membrane. The membrane was first pre-incubated for 3 hrs with a blocking solution containing 5% BSA (albumin from bovine serum, Sigma A7906) in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4). The samples were then incubated overnight with the primary antibodies (mPRα, 1:250 dilution; Pgr, 1:250 dilution; Pgrmc1, 1:250 dilution; α-tubulin (Sigma, T6074), 1:3000 dilution) in a 1% BSA blocking solution overnight. The following day, the membrane was washed five times for 5 minutes each with 1x TBST, incubated for 2 hours with horseradish peroxidase-conjugated secondary antibody (1:5000 dilutions, goat anti-rabbit antibody for mPRα, Pgr, and Pgrmc1 detection or goat anti-mouse antibody for α-Tubulin), and finally washed five times for 5 minutes each with 1x TBST. The membranes were developed using Super Signal West Extended Dura Substrate (Pierce, Rockford, IL, USA) in a plastic wrap, and then visualized using a Fluor Chem 8900 imaging station (Alpha Innotech, San Leandro, CA, USA). Protein size was determined by comparison to a biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA) and a prestained protein ladder (Fermentas). Finally, image analyzing software (ImageJ) was used to estimate relative densitometries (Schneider et al., 2012) and normalized using the expression of α-Tubulin.

Statistical analysis

All the results were analyzed using GraphPad Prism 7.0a (San Diego, CA, US) and presented as mean ± SEM. Significant differences among paired treatment groups were determined using Student's *t*-test. Gene expressions at different time points and tissues were analyzed by one-way analysis of variance (ANOVA). Two-way ANOVA was used to compare the differences

between follicular cells and denuded oocytes over different time points followed by Tukey's multiple comparisons test. Statistical significance was set at $p < 0.05$.

Results

Establishment of *pgrmc1* knockout lines in zebrafish

The first exon of *pgrmc1* contains the proper parameters for CRISPR/Cas9 target design (Figure 2.1). Successful gene editing by CRISPR was confirmed by Sanger sequencing. Several mutant lines were established; a representative line is described below. One mutant line had a deletion of 9 nucleotides and an insertion of 7 nucleotides in the *pgrmc1* gene, which led to a frameshift mutation, a premature stop codon and a truncated protein (Figure 2.2A & 2.2B). This CRISPR/Cas9-gRNA-induced specific deletion and insertion effectively disrupted the translation of *pgrmc1*, with the loss of the heme binding domain (Figure 2.2B). Using specific primers that target a frameshift site, *pgrmc1*$^{-/-}$ can be easily differentiated from *wt* using PCR (Figure 2.2C). The loss of protein was confirmed by Western blot analysis using a specific antibody against zebrafish Pgrmc1 (Figure 2.2D).

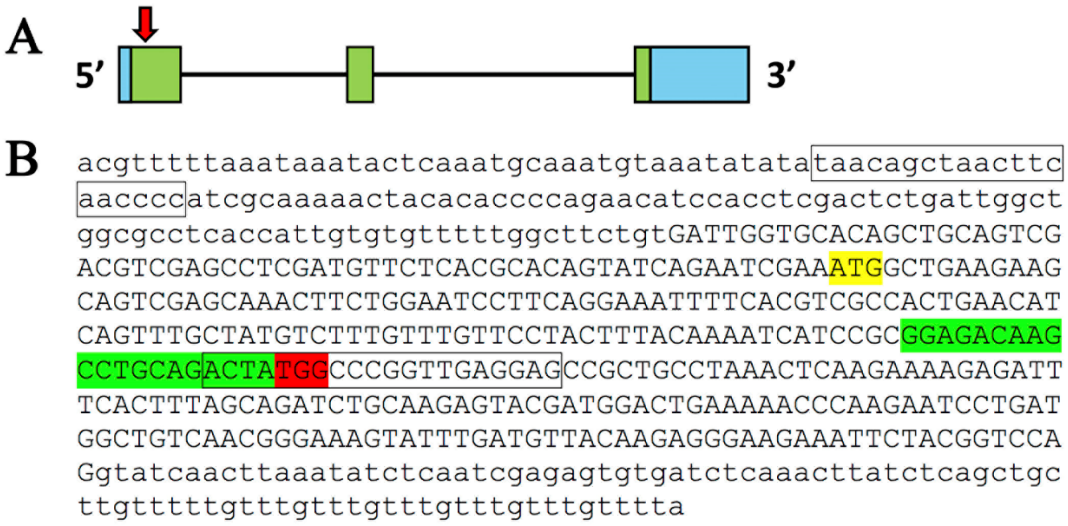


Figure 2. 1 CRISPR/Cas9 target in the *pgrmc1* gene. (A) Organization of exons and introns for *pgrmc1*. Exons are indicated by boxes; introns are indicated by black lines. Coding exons are labeled with green while untranslated regions are labeled with blue. Approximate location of the CRISPR/Cas9 target in exon 1 of *pgrmc1* is indicated by a downward red arrow**.** (B) Genomic DNA sequence of the first exon (in upper case) and flanking intron regions (in lower case) of *pgrmc1* are shown. Translation start sites (ATG) are highlighted in yellow. The sequences highlighted in red are PAM sequences and those in green are the CRISPR/Cas9 target site. Location of PCR primer for distinguishing the mutant from the wildtype is indicated with a box.

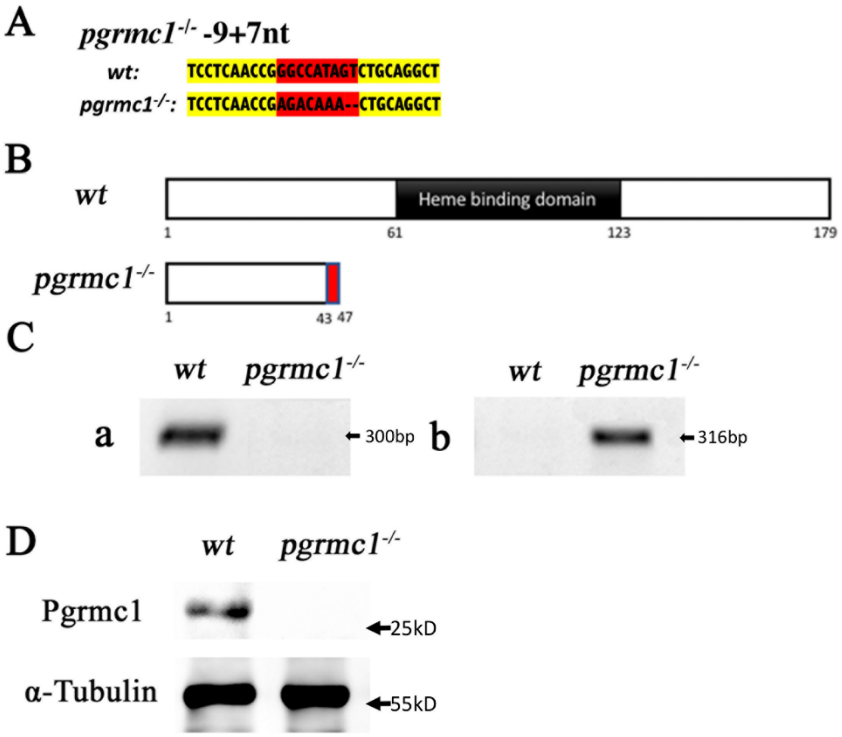


Figure 2. 2 Gene editing of *pgrmc1* in zebrafish. (A) Comparison of mutant genomic DNA sequences (small insertions or deletions) in *pgrmc1*$^{-/-}$ to those in wildtype (*wt*). The modified sequence region is highlighted in red, whereas adjacent un-modified sequences are highlighted in yellow. (B) Predicated truncated proteins from *pgrmc1*$^{-/-}$ lacking heme-binding domains. (C) Gel images of PCR products using a *pgrmc1* wildtype specific primer (a), or a *pgrmc1* mutant specific primer (b) to distinguish Pgrmc1 mutants from wildtype fish. (D) Western blot analysis confirmed the loss of Pgrmc1 protein in *pgrmc1*$^{-/-}$ stage IVa oocytes using a zebrafish Pgrmc1 specific antibody.

Reduced fertility in *pgrmc1*$^{-/-}$ female zebrafish

Expression of *pgrmc1* was found in all tissues examined, including all reproductive tissues and all stages of follicles with the highest expression in the ovaries and stage I follicles (Figure

2.3A & 2.3B). The expression of *pgrmc1* was also found in both follicular cells and denuded oocytes of stage IV follicles with no significant difference among samples collected at different times of day (Figure 2.3C).

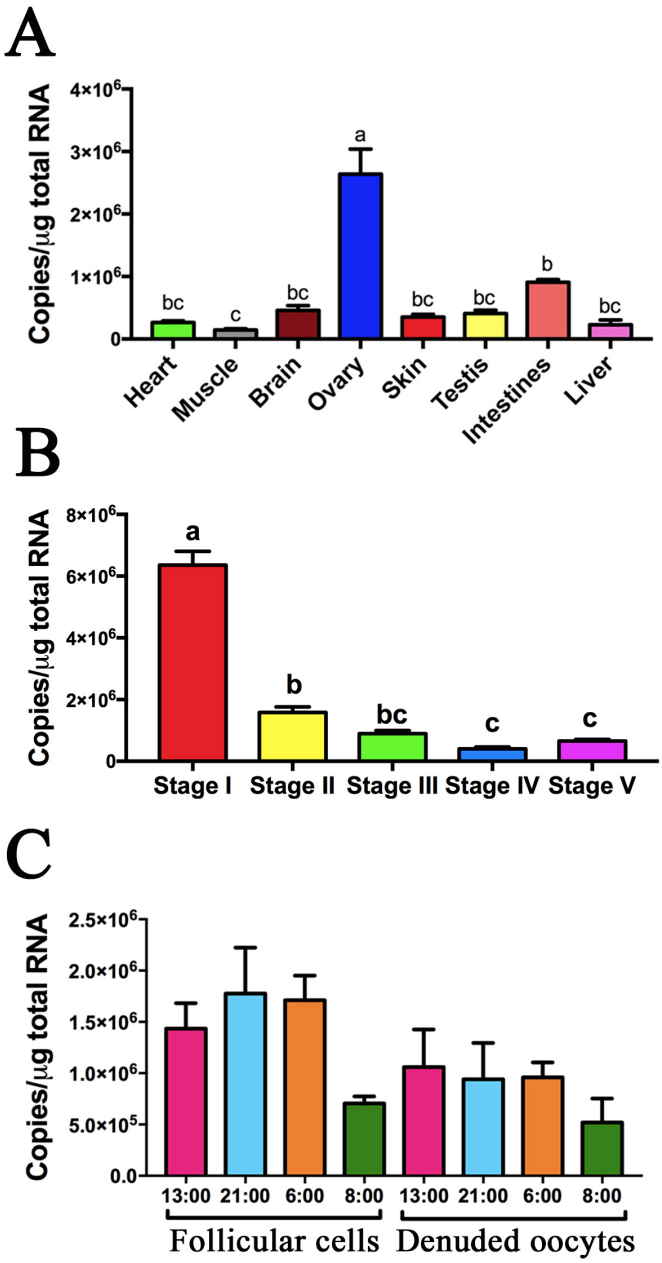


Figure 2. 3 Expression of *pgrmc1* in zebrafish. (A) Expression of *pgrmc1* in various tissue types. (B) Expression of *pgrmc1* in different stages of follicles. (C) Expression of *pgrmc1* in follicular cells and denuded oocytes.

Anatomical examination revealed no obvious differences in overall ovarian size (GSI, i.e. gonadal somatic index, Figure 2.4A) and oocyte growth or development (Figure 2.4B) between *pgrmc1*$^{-/-}$ females compared with their wildtype siblings, although more early-stage (stage I) follicles were found in *pgrmc1*$^{-/-}$ (Figure 2.4C). To further evaluate the reproductive capacities in mutant zebrafish, mature *pgrmc1*$^{-/-}$ females (at least 10 from each genotype) at 4 months of age were mated with confirmed fertile wildtype males during a minimum 4-week mating studying period. The fecundity of mutant zebrafish determined as spawning frequency, and the total number of embryos produced over two weeks following a two-week acclimation period were recorded and compared to those of wildtype crossing (wildtype males crossed with wildtype females) that were treated the same during the same period. Pgrmc1 mutant females spawned with significantly less frequency (n=11, 61.69 ± 5.30%) compared to wildtype females (n=10, 92.14 ± 1.67%) (Figure 2.4D).

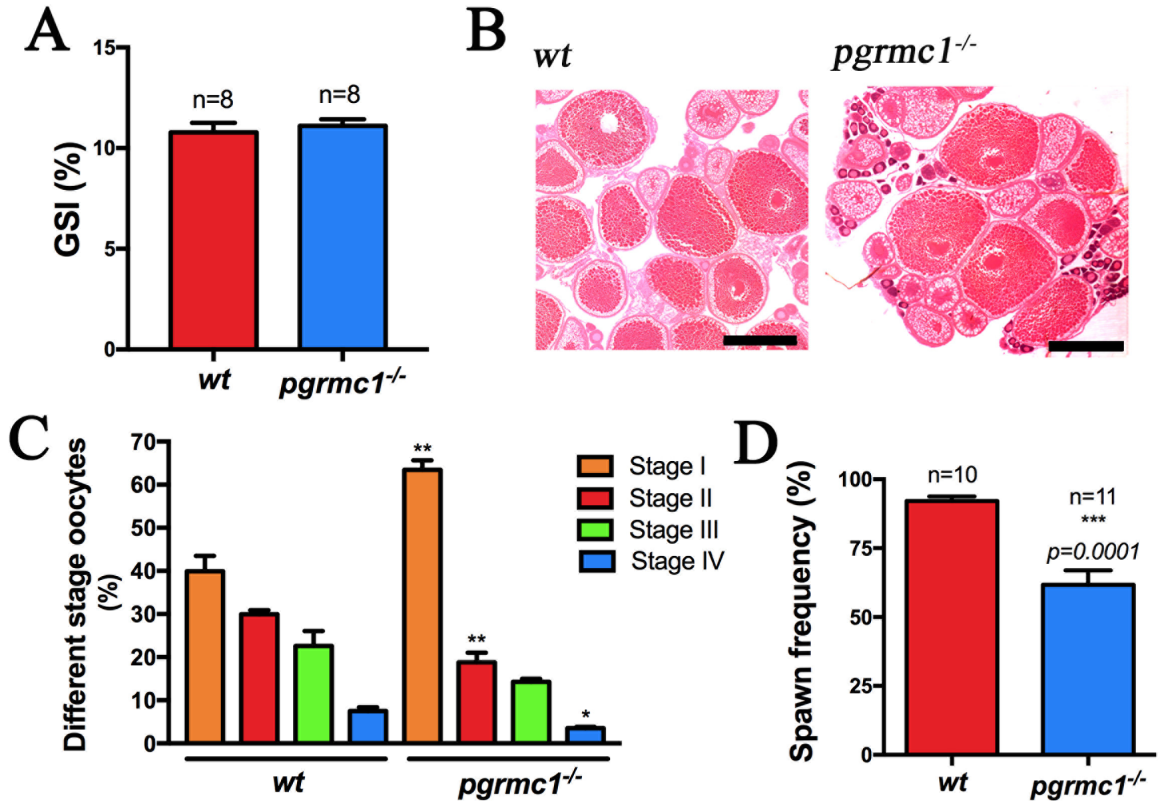


Figure 2. 4 Comparison of ovarian size, Morphology and spawning frequency between

***pgrmc1*$^{-/-}$ and wildtype.** (A) Gonadosomatic index (GSI) of wildtype (*wt*) and *pgrmc1*$^{-/-}$. (B) HE staining of a representative ovarian section from a *pgrmc1*$^{-/-}$ female showed well-formed different stages of oocytes with no obvious difference compared to wildtype (*wt*). Scale bars: 500 μm. (C) A higher percentage of stage I oocytes was found in *pgrmc1*$^{-/-}$. (D) Mutant females spawned with less frequency than wildtype. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Interestingly, *pgrmc1*$^{-/-}$ females produced a significantly lower number of live embryos daily than wildtype females over two-week continuous mating tests (Figure 2.5A & 2.5B; *pgrmc1*$^{-/-}$: 968 ± 134.8 live embryos per female in two weeks; *wt*: 1827 ± 63.2 live embryos per female in two weeks, $p<0.0001$). The lower number of live embryos produced by mutants was not due to reduced survival of embryos since similar survival rates were observed in *pgrmc1*$^{-/-}$ as in wildtype embryos (data not shown). Intriguingly, fewer follicles in *pgrmc1*$^{-/-}$ females ovulated (Figure 2.5C), which is consistent with the lower number of live embryos produced by the mutants. Fewer ovulated oocytes could be due to defects in oogenesis, follicular growth or development. However, *pgrmc1*$^{-/-}$ females have similar numbers of stages II to III follicles compared to wildtype females (data not shown). Typically, fully grown immature follicles (stage IVa) would have already completed the processes of oocyte maturation and ovulation after the lights had been on for half an hour, and no stage IVa follicles would be found in the ovaries in wildtype females (Figure 2.5C). However, a significant number of these stage IVa immature fully grown follicles could still be observed in the *pgrmc1*$^{-/-}$ (Figure 2.5C). These results indicate that the oocyte maturation process *in vivo* was affected in *pgrmc1*$^{-/-}$. Attenuated oocyte maturation also affected embryo sizes. The yolk size of each genotype was determined in sphere to 30% epiboly stage embryos. As predicted, the mean yolk size of *pgrmc1*$^{-/-}$ embryos was larger than that of wildtype individuals (Figure 2.5D), likely caused by delays in the maturation process.

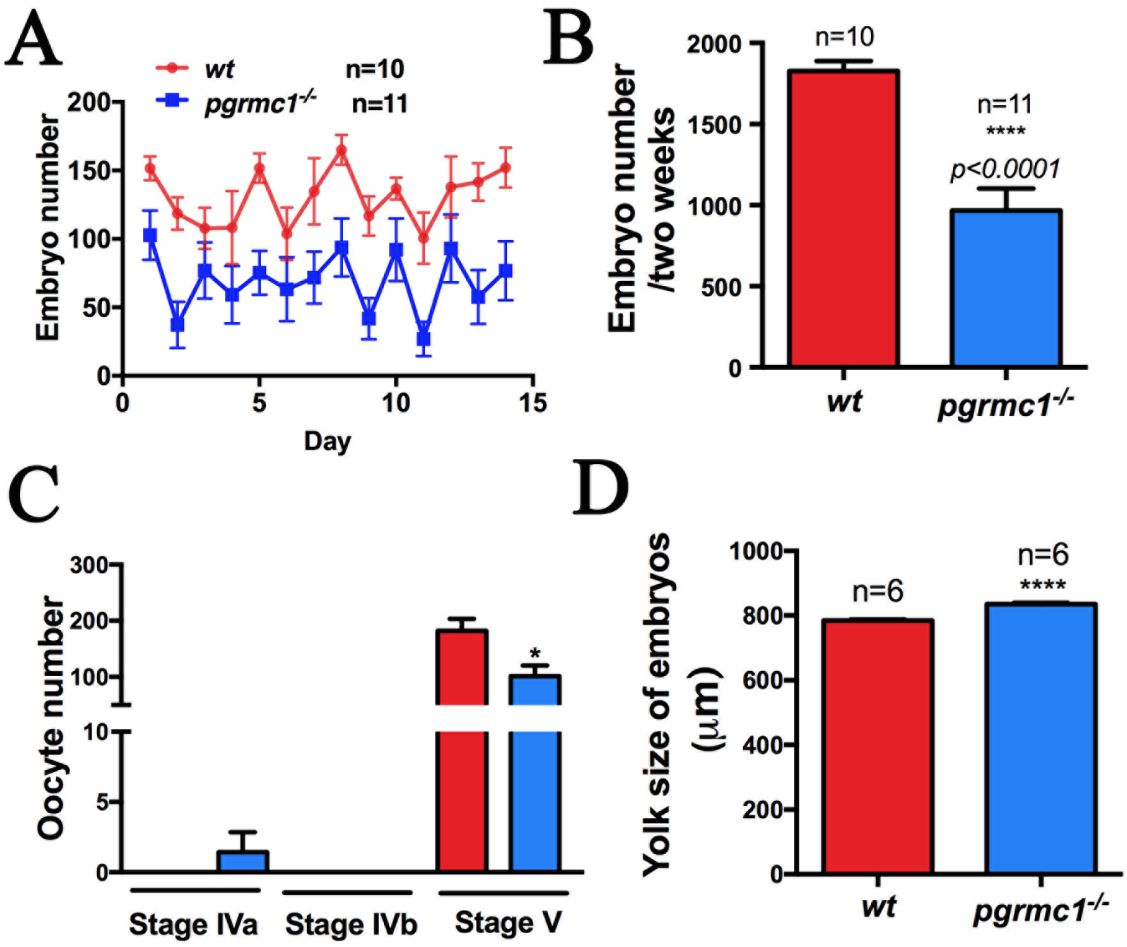


Figure 2. 5 Reduced fertility in *pgrmc1*$^{-/-}$ female zebrafish. (A) Mutant females produced fewer embryos daily than wildtype (*wt*) females. (B) Mutant female zebrafish produced fewer embryos over a two-week mating period. (C) Some leftover immature stage IVa oocytes were observed in *pgrmc1*$^{-/-}$, while none were leftover in *wt* as all these oocytes had completed maturation and ovulation before lights turned on in the morning. Consequently, fewer ovulated stage V oocytes were found in *pgrmc1*$^{-/-}$. (D) Significantly larger yolks were observed in *pgrmc1*$^{-/-}$ embryos in comparison to those from *wt*. *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$.

Reduced oocyte maturation and expression of mPRα in *pgrmc1*$^{-/-}$

To examine whether the attenuated oocyte maturation is due to down-regulation of sensitivity to progestin in the stage IVa follicles, *in vitro* GVBD tests were conducted. A slower rate of oocyte maturation and a significant decrease in the percentage of follicles that underwent maturation in response to DHP was observed in *pgrmc1*$^{-/-}$ compared to those in wildtype (Figure

2.6A).

To further elucidate the molecular mechanisms underlying the impaired oocyte maturation in the ovaries of *pgrmc1*$^{-/-}$, the expression of maturation related genes, including mPRα, were analyzed. Reduced expression of the mPRα protein was found in stage IVa follicles collected from *pgrmc1*$^{-/-}$ (Figure 2.6C), although the transcripts of mPRα were upregulated (Figure 2.6B). No significant difference was found in the expression of other related genes including mPRβ (Paqr8), mPRγ1 (Paqr5a) and mPRδ (Paqr6) in stage IVa follicles between *pgrmc1*$^{-/-}$ and wildtype (data not shown, expression of mPRγ2 (Paqr5b) or mPRε (Paqr9) were below the qPCR detection limit in 45 cycles). Moreover, lower progesterone binding was found in ovarian membranes obtained from *pgrmc1*$^{-/-}$ compared to wildtype (Figure 2.6D).

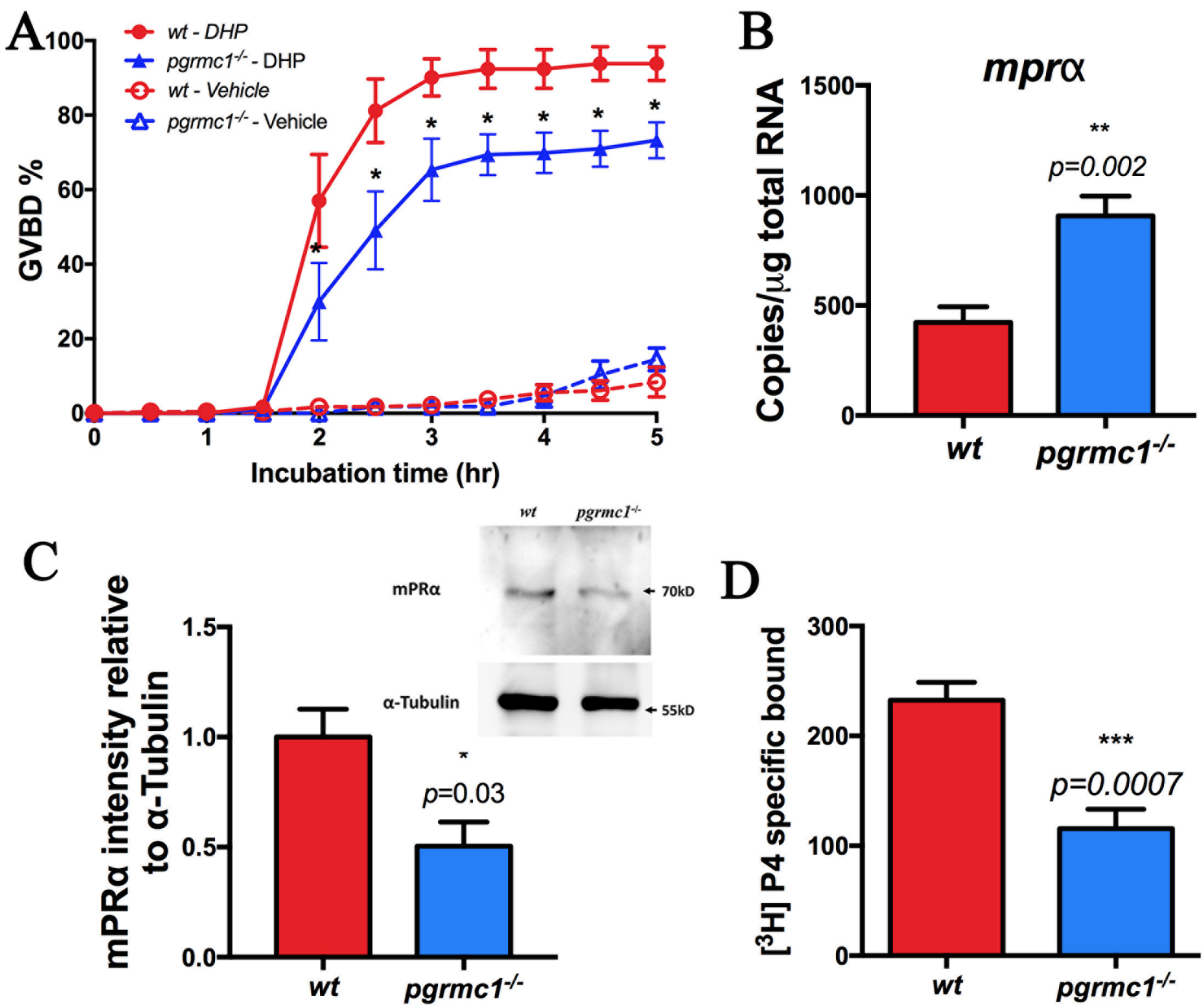


Figure 2. 6 Attenuation of oocyte maturation and reduced mPRα expression in *pgrmc1*$^{-/-}$. (A) Reduced sensitivity and oocyte maturation in response to progestin (DHP, 17α,20β-dihydroxy-4-pregnen-3-one) stimulation *in vitro* in stage IVa fully-grown immature follicles from *pgrmc1*$^{-/-}$. (B) Transcript of *mPRα* was upregulated in stage IVa follicles in *pgrmc1*$^{-/-}$. (C) Significant low level of mPRα protein in stage IVa follicles in *pgrmc1*$^{-/-}$ (n=4). Insert, a representative Western blot of proteins extracted from stage IVa follicles. (D) Lower progesterone binding was found in the *pgrmc1*$^{-/-}$ ovarian membrane compared with wildtype. n=6. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Oocyte ovulation process was not affected in *pgrmc1*$^{-/-}$

Mature, but not ovulated, follicles (stage IVb follicles) could not be found in the ovaries of *wt* and *pgrmc1*$^{-/-}$ *in vivo* 30 minutes after daily lights were turned on (Figure 2.5C). This indicates the oocyte ovulation process was not affected in mutants. Transcript and protein levels of Pgr, a

master regulator of ovulation, were not significantly different than in *wt* and *pgrmc1*$^{-/-}$ stage IVa oocytes (Figure 2.7A & 2.7B). The expression of other genes involved in ovulation, such as luteinizing hormone/choriogonadotropin receptor (*lhcgr*) and ADAM metallopeptidase domain 8b (*adam8b*) were also not affected in *pgrmc1*$^{-/-}$ (Figure 2.7C & 2.7D).

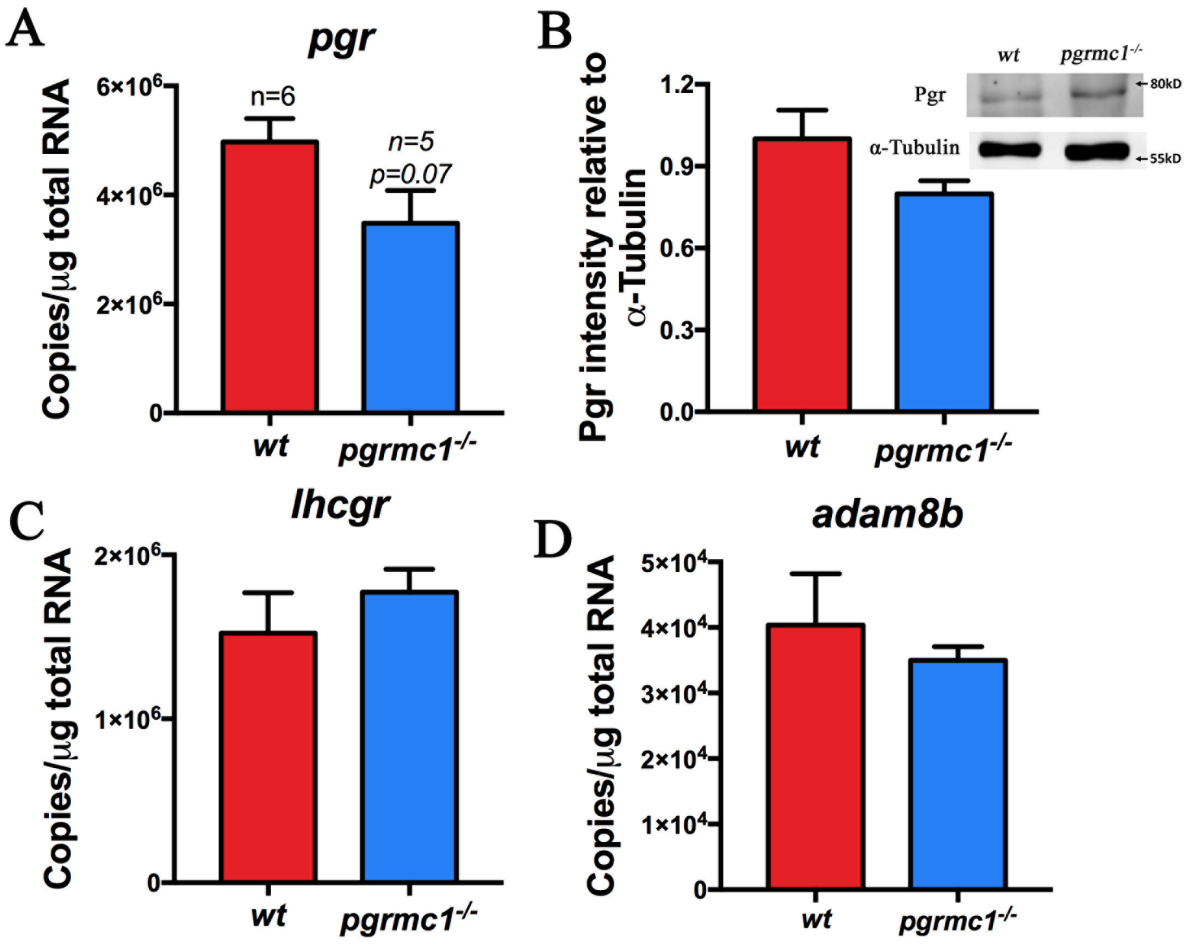


Figure 2. 7 No significant difference in the expression of *pgr*, *lhcgr*, and *adam8b*. (A) Transcript of *pgr* in stage IVa follicles in *pgrmc1*$^{-/-}$ and *wt*. (B) Pgr protein in the stage IVa follicles of *pgrmc1*$^{-/-}$ and wildtype. Insert, a representative Western blot of proteins extracted from stage IVa follicles from *pgrmc1*$^{-/-}$ or wildtype. (C, D) Transcripts of *lhcgr* or *adam8b* in stage IVa follicles in *pgrmc1*$^{-/-}$ or *wt* (n=6).

Discussion

In female vertebrates, progestin signaling is essential for gonadal development, follicular apoptosis, oogenesis, oocyte maturation, and ovulation (Nagahama and Yamashita, 2008; Peluso, 2013; Thomas, 2012; Zhu et al., 2008). The classical (genomic) mechanism of progestin action mediated through a transcription factor, the nuclear receptor, Pgr, has been extensively investigated and well-characterized (Akison and Robker, 2012; Kubota et al., 2016; Lydon et al., 1995; Zhu et al., 2015). In addition, it has become widely recognized that progestins and other steroid hormones also exert nonclassical actions involving activation of intracellular signaling pathways and rapid cellular response which is often nongenomic. However, the identities of the receptors mediating these rapid progestin actions are still the subject of active debate (Thomas, 2008, 2012; Zhu et al., 2008). To date, three candidate families of progestin receptors have been suggested to have various roles related to nonclassical progestin signaling and functions: Pgr in an extra-nuclear location (Boonyaratanakornkit et al., 2001), membrane progesterone receptors (mPRs/PAQRs,(Thomas et al., 2007; Zhu et al., 2003b)), and members of the PGRMC family (Aizen et al., 2018; Aizen and Thomas, 2015; Cahill, 2007; Lösel et al., 2008; McCallum et al., 2016; Peluso et al., 2008a; Peluso et al., 2006; Rohe et al., 2009; Sleiter et al., 2009; Thomas, 2008, 2012; Thomas et al., 2014; Zhu et al., 2008). On the other hand, cumulative evidence has indicated that PGRMC1 has numerous other functions (Cahill, 2007; Lösel et al., 2008; Thomas, 2008). For example, PGRMC1 is a heme-binding protein with a cytochrome b_5 motif, which binds and activates P450 proteins and is involved in steroidogenesis and drug metabolism (Cahill, 2007; Crudden et al., 2006; Ghosh et al., 2005; Hughes et al., 2007; Kabe et al., 2016; Mifsud and Bateman, 2002; Min et al., 2005; Min et al., 2004). Pgrmc1 has also been suggested to be an adaptor protein which facilitates membrane localization of membrane receptors, and associates

with various signaling molecules at the plasma membrane (Ahmed et al., 2010; Aizen et al., 2018; Aizen and Thomas, 2015; Cahill and Medlock, 2017; Hardt et al., 2018; Thomas et al., 2014). It is difficult to distinguish the functions of PGRMC1 from the functions of the molecules it is associated with based on traditional biochemical or pharmacological approaches. To circumvent this problem, we generated Pgrmc1 zebrafish knockouts and found normal development and growth of follicles but fewer stage V follicles (ovulated follicles) in *pgrmc1*$^{-/-}$ compared with those in wildtype. Our result indicates that Pgrmc1 has an important role in the late stages of oocyte development. One possible mechanism is *via* regulation of mPRα expression in the plasma membrane of follicles, which regulates final oocyte maturation in zebrafish (Hanna and Zhu, 2011; Zhu et al., 2003b). Mice with a Pgrmc1 conditional knockout also had fewer antral follicles (McCallum et al., 2016; Peluso, 2013). In summary, Pgrmc1 has a conserved role in the regulation of follicle development in female vertebrates.

Our *in vivo* and *in vitro* results showed that maturation of fully grown immature oocytes was significantly delayed in *pgrmc1*$^{-/-}$ zebrafish. Using biochemical and pharmacological approaches, a similar decrease in maturation in response to the progestin, DHP, was found when a Pgrmc1 inhibitor, AG205, was used in an *in vitro* zebrafish oocyte maturation assay (Aizen et al., 2018; Aizen and Thomas, 2015). Pgrmc1 is unlikely to directly regulate oocyte maturation, as there is no evidence for a direct interaction between Pgrmc1 and the inhibitory G-protein known to be important for meiosis resumption in fish. There is now extensive evidence in several teleost models that progestin induction of oocyte maturation is dependent upon a gonadotropin-dependent increase in oocyte membrane expression of mPRα (Aizen et al., 2018; Thomas, 2012; Thomas et al., 2004; Tokumoto et al., 2006; Zhu et al., 2003b). Therefore, Pgrmc1 likely facilitates oocyte maturation through regulating the oocyte plasma membrane localization of mPRα. Pgrmc1 co-

immunoprecipitates with mPRα, and manipulation of Pgrmc1 expression with siRNA or mRNA transfection alters the plasma membrane localization of mPRα in zebrafish oocytes resulting in alterations in the oocyte (Thomas et al., 2014; Thomas et al., 2007). Our recent study also indicates a close association between Pgrmc1 and mPRα, and between Pgrmc1 and Erbb2 in zebrafish oocytes at the plasma membrane of stage IV follicles using *in situ* proximity ligation assays (Aizen et al., 2018). Taken together, these results suggest that Pgrmc1 can regulate the expression and localization of proteins such as mPRα at multiple levels including transcription regulation, membrane localization, and signaling of mPRα, which in turn controls oocyte maturation. Besides mPRα, Pgrmc1 has also been suggested to be associated with chromatin and to colocalize with Aurora kinase B (Aurkb) during metaphase I and II (Luciano et al., 2010; Terzaghi et al., 2016).

Pgrmc1 was also been suggested to be involved in estrogen maintenance of zebrafish oocyte meiotic arrest, *via* regulating G protein-coupled estrogen receptor 1 (Gper) -dependent epidermal growth factor receptor (Egfr) signaling (Aizen and Thomas, 2015). The Pgrmc1 inhibitor, AG205, blocks the stimulatory effect of an Egfr inhibitor in oocyte maturation, and decreases expression of Egfr on the oocyte membrane (Aizen and Thomas, 2015). Pgrmc1 may inhibit or stimulate oocyte maturation by acting as an adaptor protein involved in membrane trafficking of mPRα, Egfr, or Erbb2 according to development stage of the oocytes and/or serum concentrations of estrogens and progestins.

It had been suggested that PGRMC1 might compensate for the loss of PGR based on higher levels of the PGRMC1 transcript found in the brains of PGR-KO female mice (Lösel and Wehling, 2003). However, no changes of PGR were observed in the ovaries of PGRMC1 knockout mice (McCallum et al., 2016). Similarly, there is no significant difference in the expression of Pgr

protein or transcript in stage IVa follicles in *pgrmc1*$^{-/-}$ in comparison with those in wildtype zebrafish, as well as the ovulation process in *pgrmc1*$^{-/-}$.

Pgrmc1 has numerous actions and is broadly distributed in zebrafish tissues and throughout the brain-pituitary-gonad axis in mammals (Liu and Arbogast, 2009). Therefore, it is likely that the Pgrmc1 knockout caused defects in physiological processes other than oocyte maturation, which may contribute to the subfertility observed. Pgrmc1 has a role in the regulation of cholesterol and steroid synthesis because of its cytochrome b5 motif and its ability to bind and activate P450 proteins (Hughes et al., 2007; Suchanek et al., 2005). Knocking out Pgrmc1 might cause deficiencies in cholesterol synthesis and increased apoptosis, which could lead to reduced fertility (Causey et al., 2011; Mansouri et al., 2008). Pgrmc1 has also been suggested to play a role in the conversion of testosterone into estrogen (Ahmed et al., 2012). Estrogen is important for vitellogenesis and growth of oocytes. Knocking out Pgrmc1 may lead to low levels of estrogen in mutants, which in turn may contribute to slow growth of oocytes and subfertility. Although we did not find obvious defects in oogenesis in Pgrmc1 knockout zebrafish, we cannot exclude a role of Pgrmc1 in steroidogenesis, which could be compensated by Pgrmc2, the paralog of Pgrmc1. Pgrmc1 likely has important reproductive functions in organs other than ovaries, such as in the hypothalamus where rapid effects of progestins on gonadotropin releasing hormone (GnRH) secretion have been demonstrated in fish (Thomas et al., 2004). There is evidence that PGR-independent rapid progestin effects in rodent GnRH-secreting cells are mediated through mPRα and mPRβ (Sleiter et al., 2009), Therefore, Pgrmc1 could potentially alter GnRH secretion through regulating the membrane expression of mPRs on GnRH-secreting cells. A possible deficiency in the synthesis and release of GnRH in the zebrafish brain induced by a Pgrmc1 deficit in the knockouts might also be a cause of subfertility. However, additional research will be required to

test this possibility. Nonetheless, results of our *in vitro* study suggest the expression of mPRα at the surface of oocytes regulated by Pgrmc1 is one important mechanism for Pgrmc1 regulation of female fertility.

In summary, Pgrmc1 is important for female fertility. Knocking out Pgrmc1 reduced fertility in female zebrafish. Pgrmc1 participates in oocyte maturation through regulation of mPR α oocyte surface expression. Our knockout also provides a model for studying functions of Pgrmc1 and its interactions with other molecules in other physiological process.

CHAPTER 3: Subfertility and Reduced Oocyte Maturation *in vivo* in Pgrmc2 Knockout Zebrafish

Wu, X.-J., Williams, M.J., Patel, P.R., Kew, K.A., Zhu, Y., 2019. Subfertility and reduced progestin synthesis in Pgrmc2 knockout zebrafish. General and Comparative Endocrinology, 113218.

Chapter summary

Progestin receptor membrane component (Pgrmc1 & 2) is a heme-binding protein. Studies on Pgrmc1 have suggested possible roles in heme binding, activation of steroid-synthesizing P450s, along with binding and transferring of membrane proteins. However, the studies of Pgrmc1's paralog, Pgrmc2 are still lacking. In order to determine the physiologic function(s) of Pgrmc2, a zebrafish mutant line (*pgrmc2*$^{-/-}$) was generated. A reduction in both spawning frequency and the number of embryos produced in female *pgrmc2*$^{-/-}$ was found. This subfertility is caused by reduced oocyte maturation (germinal vesicle breakdown, GVBD) in *pgrmc2*$^{-/-}$ *in vivo*. Nonetheless, oocytes from *pgrmc2*$^{-/-}$ had similar sensitivity to 17α,20β-dihydroxy-4-pregnen-3-one (DHP, a maturation induced progestin in zebrafish) compared with wildtype (*wt*) *in vitro*. Therefore, we hypothesized that oocyte maturation tardiness found *in vivo*, could be due to lack of progestin in *pgrmc2*$^{-/-}$. Interestingly, significant reduced expressions of hormones, receptors, and steroid synthesizing enzymes including *lhcgr*, *egfra*, *ar*, and *esr2*, *cyp11a1* and *hsd3b1* were found in *pgrmc2*$^{-/-}$ ovaries. In addition, DHP levels in *pgrmc2*$^{-/-}$ ovaries showed a significant decrease compared to those in wt. In summary, Pgrmc2 regulates female fertility, likely *via* regulation of receptors and steroids in the ovary, which in turn regulates oocyte maturation in zebrafish.

Introduction

Progestin receptor membrane component 1 (Pgrmc1) is a heme binding protein, which binds and regulates cytochrome P450 protein activities and affects steroid metabolism (Oda et al., 2011; Rohe et al., 2009; Szczesna-Skorupa and Kemper, 2011). Pgrmc1 has also been suggested as a P4 receptor, an adaptor protein transferring receptors to the cell membrane to mediate P4 signaling, or an regulator of gene and protein expression (Peluso, 2013; Thomas, 2008; Thomas et al., 2014; Wu et al., 2018; Zhu et al., 2008). In addition, PGRMC1 has also been suggested to mediate antiapoptotic and antimitotic actions of P4 in rat granulosa cells (Peluso et al., 2008a). In contrast to studies conducted in Pgrmc1, studies on Pgrmc2 are rare.

Pgrmc2, like Pgrmc1, also contains a cytochrome b5 domain, indicating that Pgrmc2 may be able to bind heme and regulate cytochrome P450 proteins. Recent studies in PGRMC2 knockout mice have suggested that knocking out PGRMC2 causes premature reproductive senescence in female mice, possibly due to failure of implantation (Clark et al., 2016). However, the conserved functions of Pgrmc2 in non-mammalian vertebrates have not been studied. The effect of Pgrmc2 on reproductive processes such as oogenesis, steroid synthesis, fertility and underlying mechanisms in basal vertebrates is unclear. In the present study, a zebrafish mutant for Pgrmc2 was generated and the functions of Pgrmc2 in reproduction was examined. We found for the first time that Pgrmc2 regulated several receptors and steroid synthesis enzymes, which in turn regulate DHP, oocyte maturation and fertility in zebrafish.

Materials and methods

Animals

The Tübingen strain of zebrafish (*Danio rerio*) used here originated from the Zebrafish

International Resource Center, and then were propagated in our lab following previously published guidelines (Zhu et al., 2015). Zebrafish were raised at 28.5 ℃ on a 14-h light, 10-h dark cycle, and were feed twice daily. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

Design CRISPR-Cas9 targets and preparation of Cas9 mRNA and sgRNAs

A 5'GG-(N_{18})-NGG3' target site in exon 1 of *pgrmc2* (GGGGCCGGTCTCGGTTTAGGCGG) was selected (Figure 3.2). Cas9 mRNA and gRNA were synthesized using a modified protocol (Jao et al., 2013). For Cas9 transcripts (nls-zCas9-nls RNA), a template plasmid (pCS2-nls-zCas9-nls) was linearized by NotI digestion, and then purified using a QIAprep column (Qiagen, Germantown, MD, USA). Capped Cas9 mRNA (nls-zCas9-nls) was synthesized using mMACHINE SP6 kit (Fisher Scientific, Hampton, NH, USA) and purified using RNeasy Mini kit (Qiagen). For gRNA, template plasmids were linearized by BamHI digestion and purified using a QIAprep column. The gRNA was generated by *in vitro* transcription using MEGAshortscript T7 kit (Fisher Scientific). RNA concentration was quantified using a Nanodrop spectrophotometer (Nanodrop 2000, ThermoFisher). The size and quality of the resulting gRNA was confirmed by electrophoresis using a 2% (wt/vol) formaldehyde agarose gel.

Establishment Pgrmc2 mutant line

To generate a founder population (F0), fertilized eggs were collected from wildtype fish within 5 min of natural spawning in their crossing tanks, which were set up the night before. One-cell stage embryos were injected using glass needles, and injection was driven by compressed N2 gas, under the control of a PV820 Pneumatic PicPump (World Precision Instrument, Florida, USA). Approximately 100 ng/µl of gRNA, 150 ng/µl of Cas9 mRNA and 0.1% phenol red were co-injected into the yolks of the embryo using a glass microcapillary pipette attached to a

micromanipulator under a stereomicroscope (Leica MZ6). To estimate mutagenesis efficiency, embryos without microinjection were designated as wildtype and used as the control group. The genomic DNA was extracted from 30 normally developing wildtype or CRISPR/Cas9-gRNA-microinjected embryos two-day post fertilization (dpf), using the HotSHOT method (Meeker et al., 2007). Mutation rates were estimated by comparing the band intensities of undigested PCR products, to the intensities of digested PCR products using T7 endonucleases I assay (Zhu et al., 2015). The PCR products were cleaned through a Qiagen column, cloned into a TA cloning vector, and potential mutant clones were selected for DNA sequencing analysis to confirm the presence of frame-shift mutation (Zhu et al., 2015).

To identify germline-transmitted mutations, remaining F0 founder embryos were raised to adulthood and outcrossed with wildtype fish. Genomic DNA from each cross was extracted from 30 randomly selected and pooled F1 embryos, and the status of the target site was analyzed *via* PCR amplification, T7 Endonucleases I assay, and DNA sequencing as described above. The remaining F1 embryos with identified frame shift mutations were raised to adulthood and were individually genotyped. Genomic DNA was extracted from part of the caudal fin of each adult fish in a 50 µl hot alkaline solution, then analyzed as stated above. Heterozygous F1 adults, that carried the same frameshift mutant alleles were crossed with each other. These crosses yielded wildtype, heterozygous, and homozygous F2 fish that were further genetically and physiologically characterized. A mutant-specific reverse primer was then designed according to the mutated sequence in CRISPR/Cas9-induced mutation (pgrmc2-Forward: 5 ′ -TCAAAAGCCTTTGTTTGGTC-3 ′ ; pgrmc2-wildtype Reverse: 5 ′ -GCAGCATGCCGCCTAAACC-3 ′ ; pgrmc2- mutant Reverse: 5 ′ -AATCTGAGACTGAGCATGCCG-3 ′). PCR condition were then optimized for efficient

identification of these specific mutations. After initial denaturation for 2 min at 94 °C, the cycling reaction was performed with the profile of 30 s at 94 °C, 30 s at 61 °C and 45 s at 72 °C for 35 cycles, followed by a 10-min extension at 72 °C with a Thermal Cycler (Eppendorf, Hauppauge, NY, USA).

Spawning and fertility

After all zebrafish lines reached their maturity at ~ 4-months of age, at least 10 homozygous mutant female fish were crossed with wildtype fertility confirmed males. Production of the offspring for each genotype was recorded daily for a period of two weeks, following a two-week acclimation period. Spawning frequency is defined as the number of times a female would produce fertilized embryos in a two-week examination period.

Yolk width measurement

In total, 7 individuals of *wt* or *pgrmc2*$^{-/-}$ female fish were used and paired with wildtype males. Width of the yolk was defined as the length between two tips of the yolk. All Embryos (~100 embryos/pair) from each pair were collected in a petri dish and the diameter of the yolks was measured and recorded using a stereo microscope (SZX7, Olympus, Japan). To reduce variation, yolk width was determined at 30% epiboly stage.

Follicle isolation and quantification

Oocyte maturation in zebrafish typically occurs prior to the onset of (day) light, while ovulation and spawning occurs within 1 hour following the onset of light. At 09:30 AM, thirty minutes after laboratory lights were turned on, seven adult female fish were placed in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes, and then the spinal cord and blood supply was severed using IACUC approved procedures. The ovaries of each fish were then immediately dissected out and rinsed in 60% L-15 media (Sigma-Aldrich, St. Louis, MO, USA) containing 15

mM HEPES (pH=7.2). The term "follicles" refers to follicular cells enclosed oocytes. Follicles of various sizes were isolated from the ovaries and the diameter of each follicle was measured under a stereo microscope (SZX7, Olympus, Japan). The developmental stages of follicles were divided into five different stages based on follicular size, morphological criteria, physiological and biochemical characteristics (Selman et al., 1993; Tyler and Sumpter, 1996) with a slight modification. Stage I (<140 μm) and II (140-340 μm) follicles are previtellogenic follicles; stage III are early vitellogenic follicles (340-690 μm); stage IV are late vitellogenic follicles (690-730 μm) that are further divided into IVa and IVb two stages, IVa is maturational competent fully grown but immature follicles (IVa), IVb are matured follicles that undergo oocyte maturation but haven't undergone ovulation (IVb); and stage V ovulated follicles are ovulated eggs with no follicular cells attached (730-750 μm).

Oocyte maturation assay

Ovarian follicles were isolated and incubated in order to determine the sensitivities of follicles to a maturation inducing steroid, DHP (Hanna and Zhu, 2011; Pang and Thomas, 2009). Gravid female zebrafish were euthanized and their ovaries were immediately dissected out. Then, the ovaries were washed several times in 60% Leibovitz L-15 medium (Sigma-Aldrich). The individual ovarian follicles were carefully separated without damaging the follicular cell layers based on previously established protocols (Hanna and Zhu, 2011). Fully-grown immature oocytes were randomly selected and distributed into the wells of a 24-well plate (~30 follicles/well). Final concentration for DHP and ethanol in the treatment group was 5 nM and 0.1%, respectively. As a control, 1 μl pure ethanol was added into control wells containing 1 ml medium and same number of follicles (~30 follicles/well) collected at the same time from the same group of individual fish. The follicles were incubated in vehicle or DHP for 5 hours, with GVBDs being scored every half

hour during the incubation period. In order to confirm the results, all the experiments were repeated five times.

RNA isolation and real-time quantitative PCR

Brain, liver, and ovary tissues were quickly dissected out of mature fish around 5:30am prior to oocyte maturation and homogenized immediately in 500 µl of RNAzol reagent (Molecular Research Center, Cincinnati, OH, USA). The homogenized samples were preserved in −80 °C freezer until RNA extraction. Total RNA was isolated based on a modified protocol (Liu et al., 2017). The amount and purity of the RNA was determined using a Nanodrop 2000 (Thermo Fisher). The cDNAs were synthesized using 500 ng total RNA and High Capacity cDNA Reverse Transcription kit (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using the SYBR green with C1000 Touch Thermal Cycler (Bio-Rad). The protocol consisted of a cycling profile of 30 s at 95 °C, 30 s at 58 °C, and 45 s at 72 °C for 45 cycles followed by a melting curve test. PCR efficiency was calculated using the efficiency equation (EFF) = 10(−1/slope) − 1, and the authentic PCR products were confirmed by analyses of melting curve, gel electrophoresis, and DNA sequencing. Because the expressions of house-keeping-gene were variable in different developmental stages of oocytes, the pgrmc2 transcript was determined by the absolute transcript. The pgrmc2 transcript was determined using Ct-values of samples and standard curves generated from known serial diluted plasmid concentrations (10^2–10^7 copies/µL). The comparative Ct method was used for various gene expression in brains, livers and ovaries, and the data was normalized using *gapdhs* and expressed as fold differences of target gene expression in *pgrmc2*$^{-/-}$ relative to those in the wildtype control. The full names of the genes and the primers used in this study are listed in Table 2. 1.

DHP measurement

Ovaries were collected from 4-month old healthy and mature *wt* or *pgrmc2*$^{-/-}$ at 5:30 am prior to oocyte maturation. Immediately, collected ovaries were sonicated (Sonic Dismembrator, Fisher Scientific) in 600 µl of optima grade water (Fisher Scientific, Fairlawn, NJ) on ice. Samples were then stored in −80 °C until the analysis. Steroids were extracted from the samples using a liquid–liquid extraction (LLE) method. Briefly, 2.4 ml extraction solvent (methanol: water in 1:1 ratio containing 0.1% formic acid) was added to each sample and vortexed for 5 min. The mixture was centrifuged for 15 min at 14,000 g, and the supernatant (organic phase) was transferred into a 16 × 125mm borosilicate glass tube (VWR, Radnor, PA). Then, 3 ml of methyl-tert-butylether (Fisher Scientific) was added to the supernatant. The mixture was vortexed for 15 min and centrifuged for two minutes at 800 g. The organic phases on the top was collected. The extraction process was repeated twice in the remaining aqueous phase. The collected organic fractions from three ovarian samples were combined in the same tube, and was dried with N2 gas at a room temperature. Each sample was resuspended in 100 µl of sample buffer (70 acetonitrile:30 water containing 0.1% formic acid) for liquid chromatography/mass spectrometry (LCMS) analysis.

To determine the DHP level in samples, an external standard calibration curve was established using a serial of known concentration (0.005, 0.01, 0.05, 0.1, 0.5 µg/ml) of 17α,20β-dihydroxy-4-pregnen-3-one (DHP, Sigma-Aldrich, St. Louis, Missouri). Extracted steroids were identified using an Eskigent 425 microLC/SCIEX 5600 + Triple time-offlight mass spectrometer. Samples and standards in autosampler *via*ls were loaded in a refrigerated holder (4 °C). A HALO C18, 2.7 µm, 0.5 × 50 mm microLC column purchased from Eksigent was maintained at 25 °C. The flow rate was 10 µl/min and 5 µl of sample was injected. Mobile phase A: water with 0.1% formic acid and mobile phase B: acetonitrile. Independent data acquisition was utilized to collect

the top 20 MS/MS. For data quantification, the integration of peak areas was conducted using PeakView and MultiQuant (SCIEX), and an external standard calibration curve was used to calculate the DHP amount and normalized by the ovary weight.

Statistical analysis

All graphs were generated and results were analyzed using GraphPad Prism 7.0a (San Diego, CA, US). Significant differences among paired treatment groups were determined using Student's t-test with a significance level of $p<0.05$. Gene expressions at different stage of oocytes and in different tissues were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Lastly, a paired t-test was used to compare the differences between follicular cells and denuded oocytes across different time points.

Results

High expression of *pgrmc2* in ovary and stage I follicles

Expression of *pgrmc2* was found in all examined tissues, including all reproductive tissues and all developmental stages of follicles, with highest expression of *pgrmc2* observed in the ovaries and stage I follicles (Figure 3.1A & 3.1B). In follicles, *pgrmc2* is more highly expressed in denuded oocytes than in follicular cells (Figure 3.1C).

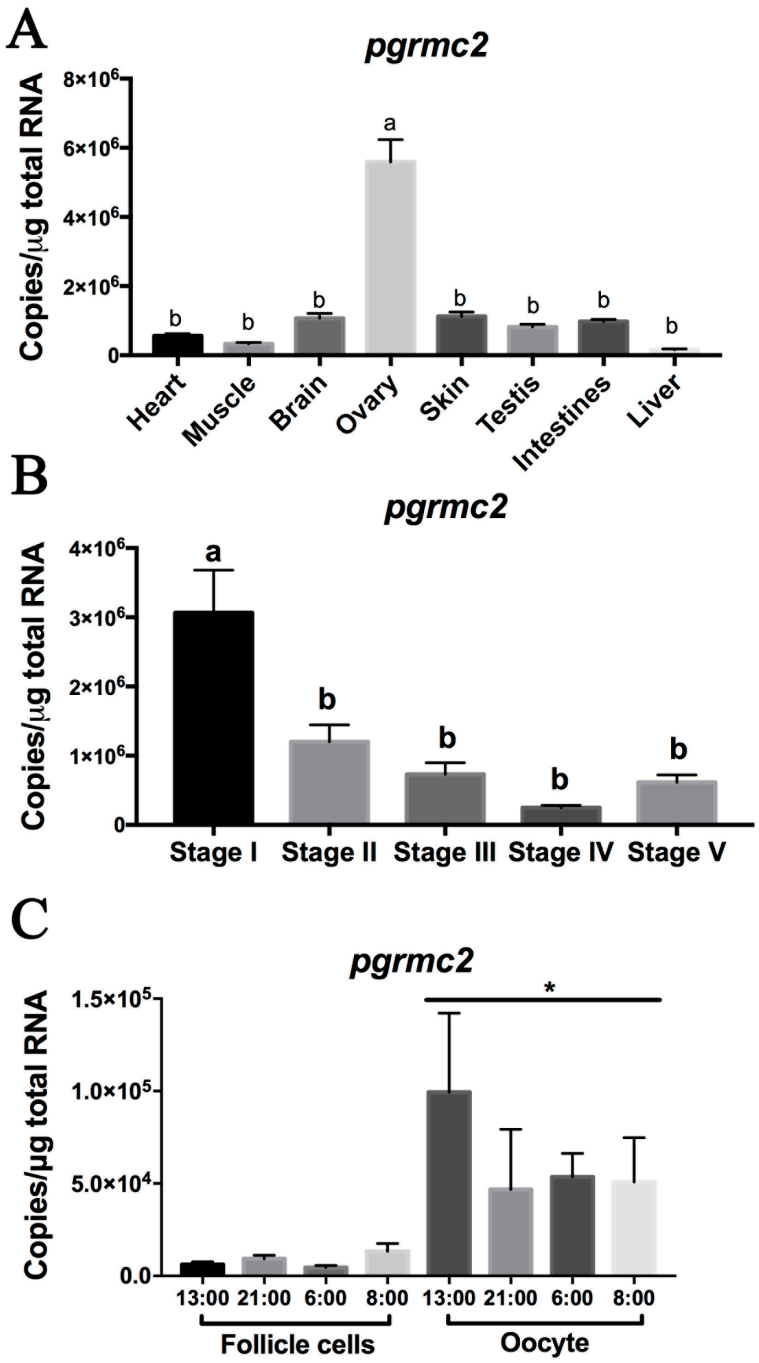


Figure 3. 1 Expression of *pgrmc2* transcripts in zebrafish. (A) Expression of *pgrmc2* in various tissue types, with the highest expression level seen in the ovary. (B) Expression of *pgrmc2* in different follicle stages. Stage I oocytes showed highest *pgrmc2* expression. (C) Expression of *pgrmc2* in follicular cells and denuded oocytes. Bars with different letters indicated significant difference. Significant difference was observed between follicle cells and denuded oocytes. *, $p<0.05$.

Establishment of Pgrmc2 mutant line in zebrafish

To investigate Pgrmc2's functions *in vivo*, we targeted the first exon of Pgrmc2 as it contains the proper parameters for CRISPR/Cas9 target design (Figure 3.2A). Successful editing was confirmed by Sanger sequencing. One mutant line had 10- and 20-nucleotide insertions in two different loci of Pgrmc2, which caused a translational frameshift in *pgrmc2* mRNA and an early stop codon, resulting in a truncated protein (Figure 3.2B & 3.2C). CRISPR/Cas9-gRNA induced specific insertions effectively disrupting the translation of Pgrmc2 and led to the loss of heme binding domain (Figure 3.2C). Using specific primers that target a frameshift site, *pgrmc2*$^{-/-}$ can be easily differentiated from wildtype using PCR (Figure 3.2D).

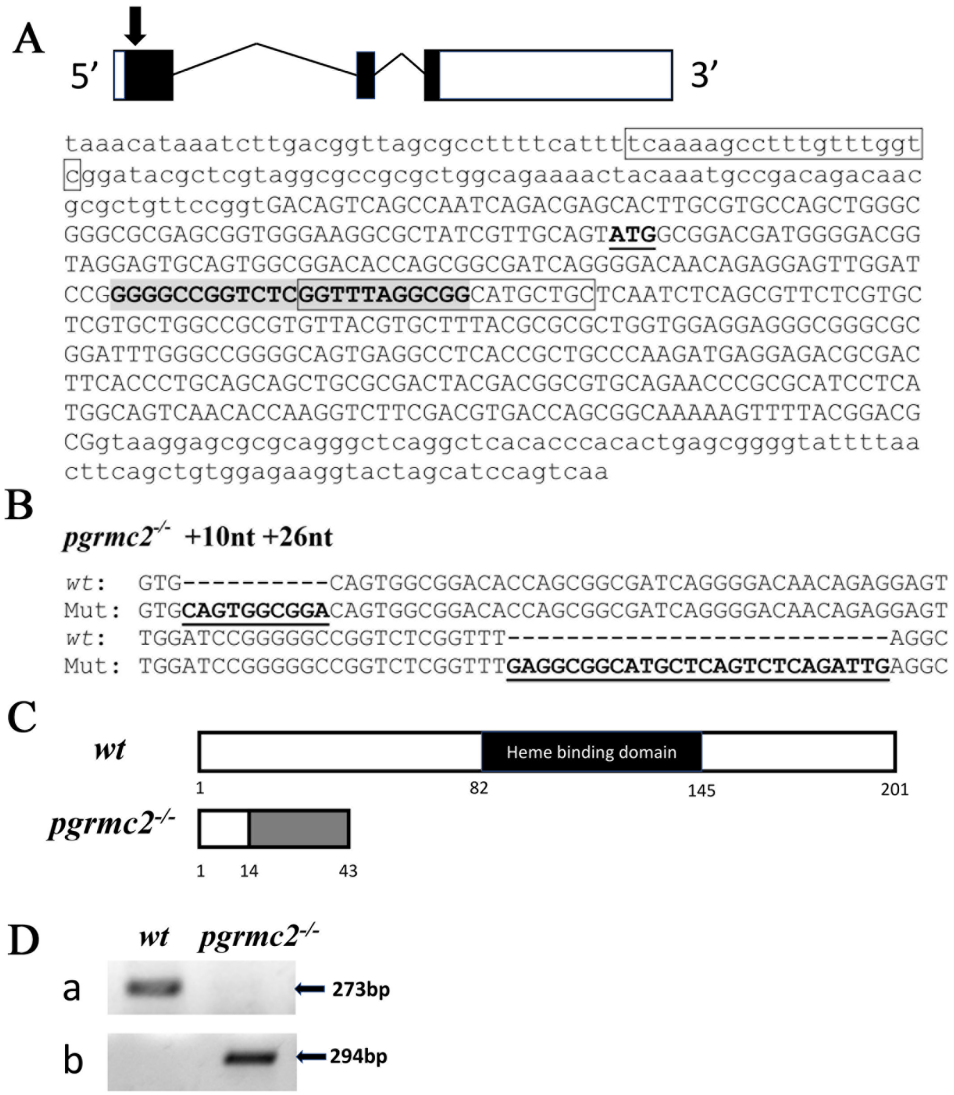


Figure 3. 2 Generate Pgrmc2 mutant line in zebrafish. (A) Organization of three exons and two introns for Pgrmc2 and the CRISPR/Cas9 target site. Exons are indicated by boxes; introns are indicated by black lines. Coding exons are labeled with filled black box, while untranslated regions are labeled with open box. Approximate location of the CRISPR/Cas9 target in exon 1 of pgrmc2 is indicated by a downward black arrow. In addition, genomic DNA sequence of first exon (in upper case) and flanking intron regions (in lower case) of Pgrmc2 are shown. Translation start sites (ATG) are indicated by bolded and underlined font. The sequence highlighted in grey is the CRISPR/Cas9 target site with a PAM motif (CGG). Location of PCR primers for distinguishing the mutant from the wildtype is indicated with two open boxes. (B) Comparison of mutant genomic DNA sequences (small insertions) in *pgrmc2*$^{-/-}$ to those in wildtype (*wt*). Modified sequence regions are bolded and underlined. (C) Schematic drawings show wildtype Pgrmc2 protein and predicate truncated proteins from *pgrmc2*$^{-/-}$ lacking heme binding domains. Small insertions in the pgrmc2 coding region, resulting in a premature stop codon in the Pgrmc2 protein. (D) Gel images of PCR products using a Pgrmc2 wildtype specific primer (a), or a Pgrmc2 mutant specific primer (b) to distinguish *pgrmc2*$^{-/-}$ from wildtype fish.

Reduced fertility in *pgrmc2*$^{-/-}$ female zebrafish

No significant difference in ovarian size was observed in *pgrmc2*$^{-/-}$ females compared with their wildtype siblings (Figure 3.3A). To evaluate the fertility in mutant zebrafish, mature *pgrmc2*$^{-/-}$ females (n=11) at 4 months of age, were mated with known fertile wildtype males during a minimum 4-week mating study period. The embryo numbers were recorded daily for two weeks and compared to those in wildtype crossing (wildtype males crossed with wildtype females, n=10) following a two-week adaptation period. Interestingly, *pgrmc2*$^{-/-}$ females (n=11, 1077 ± 127.2 embryos/fish/two weeks, $p=0.0001$) produced a significantly lower number of embryos in comparison with wildtype females (n=10, 1827 ± 63.2 embryos/fish/two weeks) (Figure 3.3B). The *pgrmc2*$^{-/-}$ females also spawned significantly less frequently (n=11, 52.6 ± 4.65%) than wildtype females (n=10, 92.14 ± 1.67%) (Figure 3.3C). Overall, wildtype females produced more embryos daily than *pgrmc2*$^{-/-}$ females over two weeks of continuous mating tests (Figure 3.3D). Histology analysis showed *pgrmc2*$^{-/-}$ ovaries have all stages of oocytes with a significantly higher percentage of stage I follicles compared to that of wildtype (Figure 3.3E & 3.3F). High ratio of stage I oocytes skewed the over proportions and resulted in lower percentages of late stage follicles in *pgrmc2*$^{-/-}$ compared to wildtype (Figure 3.3F).

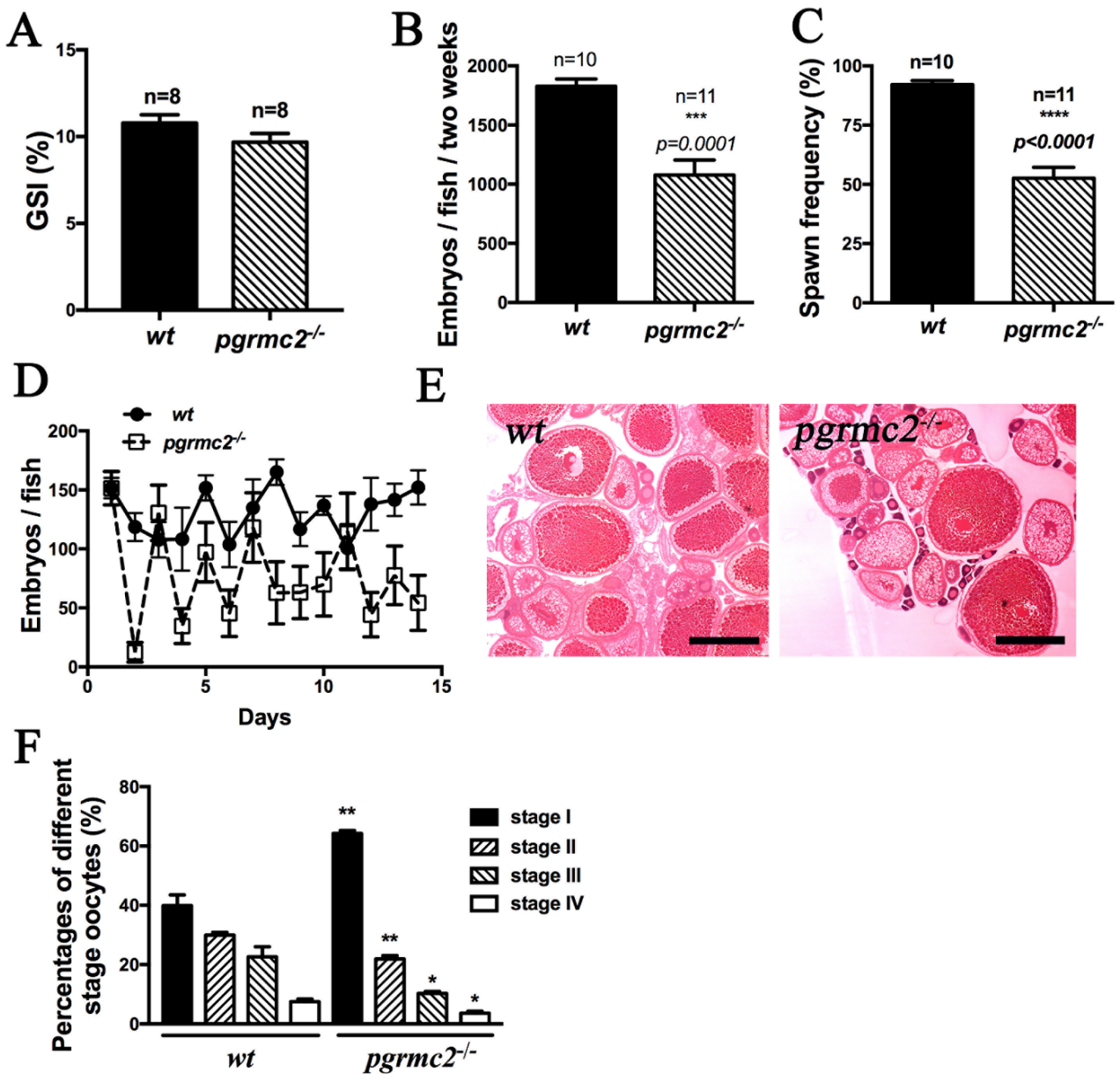


Figure 3. 3 Comparison of ovarian size, fertility and morphology between *pgrmc2*$^{-/-}$ and wildtype. (A) Comparison of ovarian size, gonadosomatic index (GSI) between *pgrmc2*$^{-/-}$ and wildtype. (B) Mutant *pgrmc2*$^{-/-}$ female zebrafish produced fewer embryos over a 2-week mating period. (C) Mutant *pgrmc2*$^{-/-}$ female zebrafish spawned with less frequency during the mating period. (D) Mutant females produced fewer embryos daily than wildtype females. (E) Morphology comparison of the ovaries. HE staining of a representative ovarian section from a *pgrmc2*$^{-/-}$ female showed well-formed different stages of oocytes with a significantly higher number of stage I follicles compared to wildtype. Scale bars: 500μm. (F) A higher number of stage I follicles, but a lower number of late stage follicles were observed in *pgrmc2*$^{-/-}$ compared to wildtype. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Maturation tardiness *in vivo* but not *in vitro* in *pgrmc2*$^{-/-}$

One possible cause for the reduced fertility found in *pgrmc2*$^{-/-}$ is deficiencies in oogenesis. Ovaries were sampled half-hour after lights on and counted the numbers of follicles in different stages. No significant differences were found in the number of early stage follicles (stage II and stage III) in the ovaries from *pgrmc2*$^{-/-}$ females compared to wildtype (Figure 3.4A). Typically, fully grown immature follicles (stage IVa) would have already successfully completed the processes of oocyte maturation and ovulation. As expected, no stage IVa was observed in the ovaries from wildtype females (Figure 3.4A). However, a significant number of stage IVa follicles could still be observed in the *pgrmc2*$^{-/-}$ females (Figure 3.4A). These results suggest reduced oocyte maturation in *pgrmc2*$^{-/-}$ *in vivo*.

One possible cause for reduced oocyte maturation is reduced progestin sensitivity due to reduced expressions of membrane progestin receptors. However, fully grown but immature follicles from *pgrmc2*$^{-/-}$ had normal sensitivity to DHP (Figure 3.4B), as compared to those from wildtype females. Oocytes from *pgrmc2*$^{-/-}$ matured at the same rate as wildtype over the 5 hour DHP treatment. In addition, no significant differences were found between wildtype and *pgrmc2*$^{-/-}$ in the expression of maturation related genes including *mprγ* (*Paqr5a*), *mprδ* (*paqr6*), *mprα1* (*paqr7a*), *mprα2* (*paqr7b*), and *mprβ* (*paqr8*) in the ovaries (Figure 3.4C). Expressions of *mprγ2* (*paqr5b*) and *mprε* (*paqr9*) were under qPCR detection limit (in 45 cycles) in stage IVa follicles from wildtype and *pgrmc2*$^{-/-}$. The yolk sizes of 30% epiboly stage embryos were also measured. The mean yolk size of *pgrmc2*$^{-/-}$ embryos was similar to that of wildtype individuals (Figure 3.4D). However, *pgrmc2*$^{-/-}$ had a large number of extremely small sized embryos (<600 μm), all of which died by 4-day post fertilization. These results indicated that, the attenuated oocyte maturation

found *in vivo* was not due to down-regulation of membrane progestin receptors in the stage IVa follicles of *pgrmc2*$^{-/-}$.

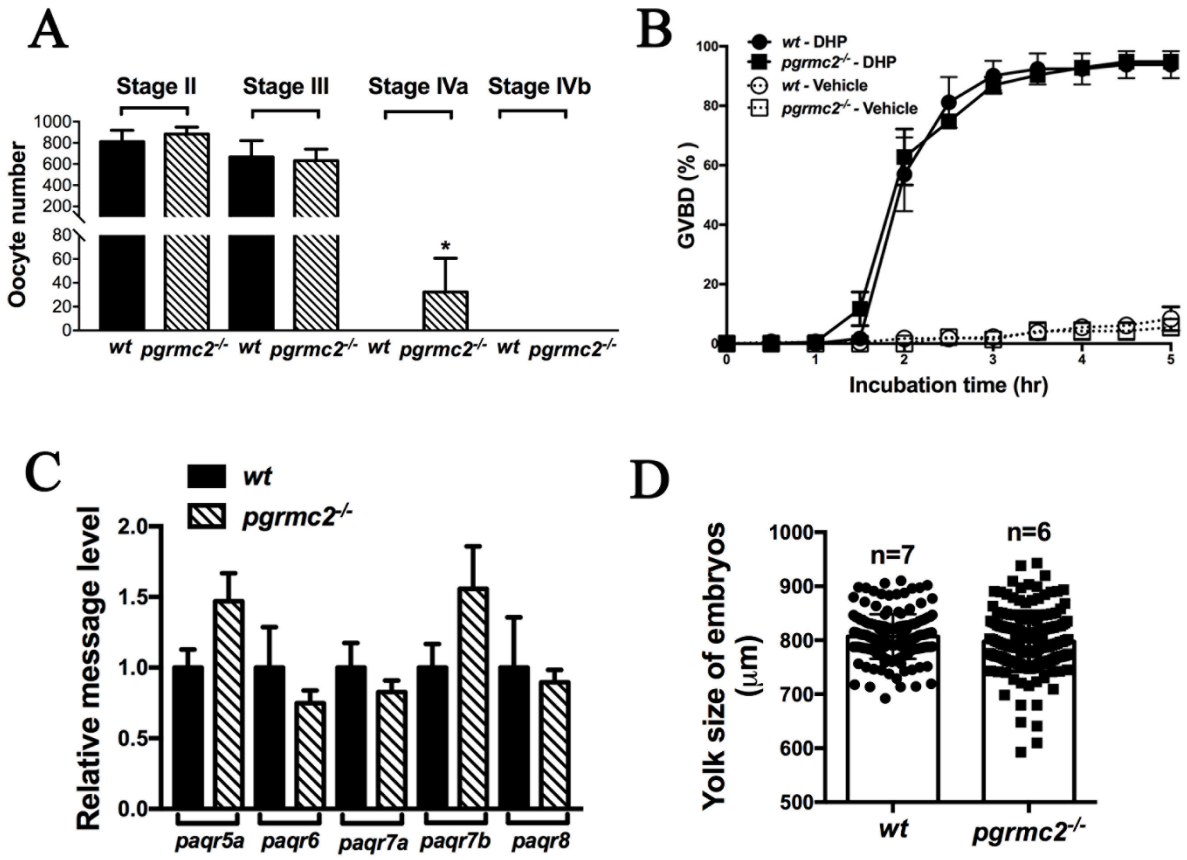


Figure 3. 4 Attenuation of oocyte maturation *in vivo* but not *in vitro* in *pgrmc2*$^{-/-}$. (A) Some leftover immature stage IVa oocytes were observed in *pgrmc2*$^{-/-}$ 30 minutes after room lights were switched on, while no stage IVa remained in *wt*, because all of them had matured and ovulated before lights were switched on that morning. There was no difference in terms of stage II and stage III oocyte number. (B) Similar sensitivity and oocyte maturation in response to progestin (DHP, 17α,20β-dihydroxy-4-pregnen-3-one) stimulation *in vitro* in fully-grown immature follicles from *pgrmc2*$^{-/-}$. (C) No difference of transcript of mPRs in stage IVa follicles in *pgrmc2*$^{-/-}$ compared to *wt*. (D) Similar yolk sizes were observed in *pgrmc2*$^{-/-}$ embryos in comparison to those from *wt*. *, $p<0.05$.

Downregulation of genes that are essential for progestin synthesis

Thereafter, we hypothesized that reduced oocyte maturation *in vivo* is due to reduced progestin synthesis. In *pgrmc2*$^{-/-}$ adult female brains, follicle stimulating hormone subunit beta (*fshb*) and 3β-hydroxysteroid dehydrogenase (*hsd3b1*) were downregulated (Figure 3.5A). In liver,

the gene expression of the insulin-like growth factor 1 (*igf1*) was significantly lower in *pgrmc2*$^{-/-}$ than *igf1* expression in wildtype (Figure 3.5B). Other genes that are important for cholesterol synthesis were expressed similarly to those in wildtype in the liver. Interestingly, significant down-regulation of steroidogenic enzymes including *cyp11a*, *hsd3b1*, *cyp17a1*, and *hsd17b1* were observed in the *pgrmc2*$^{-/-}$ ovaries. *Cyp11a* and *hsd3b1* are both essential for progesterone synthesis while *cyp17a1* and *hsd17b1* are important for testosterone and estradiol synthesis (Figure 3.6). In addition, low expression of luteinizing hormone-choriogonadotropin receptor (*lhcgr*), androgen receptor (*ar*), estrogen receptor 2 *(esr2,* including *esr2a* and *esr2b*), epidermal growth factor receptor a *(egfra)* were also observed in the *pgrmc2*$^{-/-}$ovaries.

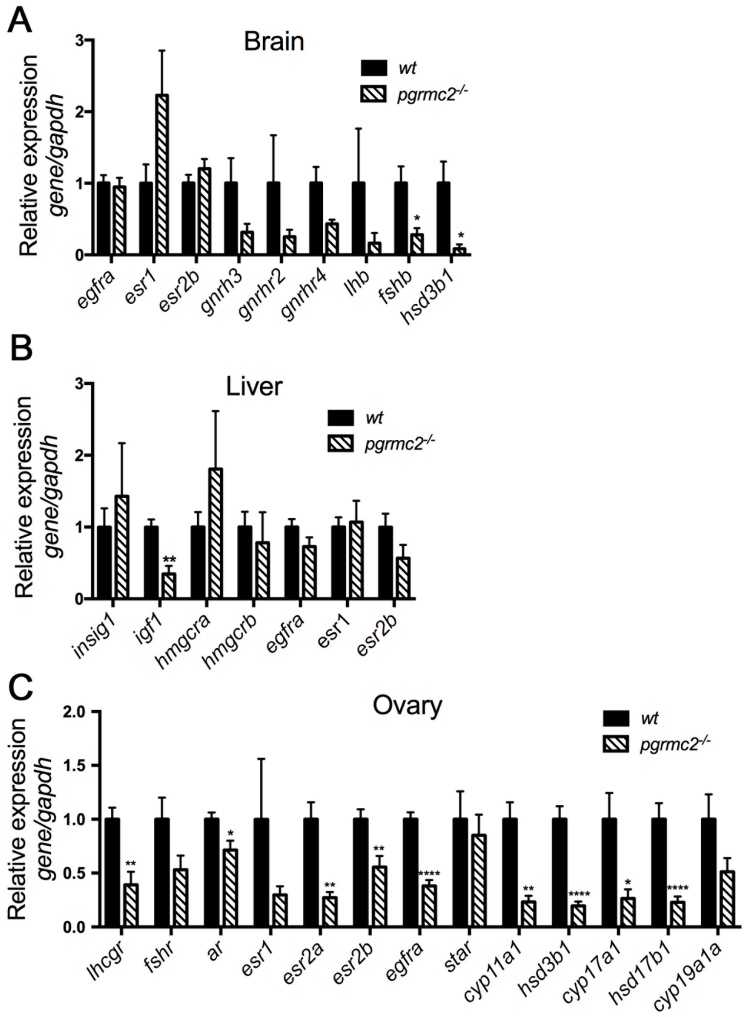


Figure 3. 5 Gene expression difference between wildtype and *pgrmc2*$^{-/-}$ females in brain (A), liver (B) and ovary (C). (A) In brain, *fshb* and *hsd3b1* are downregulated. (B) *igf1* is lower in liver of *pgrmc2*$^{-/-}$ and the genes involved in cholesterol synthesis show no change. (C) In the ovary, many genes express less in the *pgrmc2*$^{-/-}$, especially the steroid-synthesis enzymes. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. (*ar*: androgen receptor; *cyp11a1*: cytochrome P450 side-chain cleavage; *cyp17a1*: cytochrome P450 family 17 subfamily A member 1; *cyp19a1a*: cytochrome P450, family 19, subfamily A, polypeptide 1a; *egfra*: epidermal growth factor receptor a; *esr*: estrogen receptor; *fshb*: follicle stimulating hormone subunit beta; *fshr*: follicle stimulating hormone receptor; *gnrh*: gonadotropin-releasing hormone; *gnrhr*: gonadotropin-releasing hormone receptor; *hmgcr*: 3-hydroxy-3-methylglutaryl-CoA reductase; *hsd3b1*: 3β-hydroxysteroid dehydrogenase; *hsd17b1*: 17β-hydroxysteroid dehydrogenase; *igf1*: insulin-like growth factor 1; *insig1*: insulin induced gene 1; *lhb*: luteinizing hormone beta; *lhcgr*: luteinizing hormone receptor; *star*: steroidogenic acute regulatory protein).

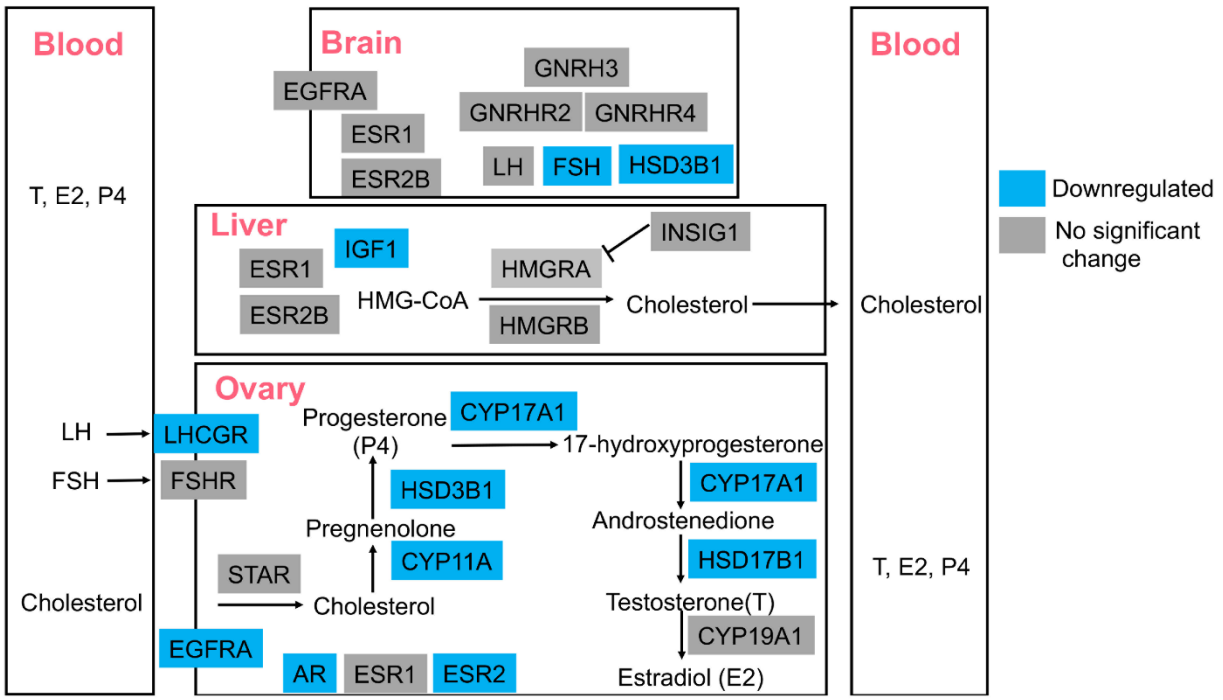


Figure 3. 6 Reproduction-related gene expression profile in adult *pgrmc2*$^{-/-}$ female zebrafish in comparison to those in wildtype along the brain-liver-gonad axis. Using expression in the wildtype as reference, significantly down-regulated genes are marked in blue; genes with no statistically significant change are marked in grey. (*ar*: androgen receptor; *cyp11a1*: cytochrome P450 side-chain cleavage; *cyp17a1*: cytochrome P450 family 17 subfamily A member 1; *cyp19a1a*: cytochrome P450, family 19, subfamily A, polypeptide 1a; *egfra*: epidermal growth factor receptor a; *esr*: estrogen receptor; *fshb*: follicle stimulating hormone subunit beta; *fshr*: follicle stimulating hormone receptor; *gnrh*: gonadotropin-releasing hormone; *gnrhr*: gonadotropin-releasing hormone receptor; *hmgcr*: 3-hydroxy-3-methylglutaryl-CoA reductase; *hsd3b1*: 3β-hydroxysteroid dehydrogenase; *hsd17b1*: 17β-hydroxysteroid dehydrogenase; *igf1*: insulin-like growth factor 1; *insig1*: insulin induced gene 1; *lhb*: luteinizing hormone beta; *lhcgr*: luteinizing hormone receptor; *star*: steroidogenic acute regulatory protein).

Reduced progestin in *pgrmc2*$^{-/-}$ ovaries

To further elucidate why oocyte maturation delay happened *in vivo* but not *in vitro* in the ovaries of *pgrmc2*$^{-/-}$, the progestin (DHP) content in ovaries was analyzed using LCMS. As expected, the DHP level in the ovaries from *pgrmc2*$^{-/-}$ (0.076 ± 0.016 µg/g ovary, $n = 4$) was significantly lower than that in wildtype (0.30 ± 0.04 µg/g ovary, $n = 4$) ($p < 0.01$) (Figure 3.7).

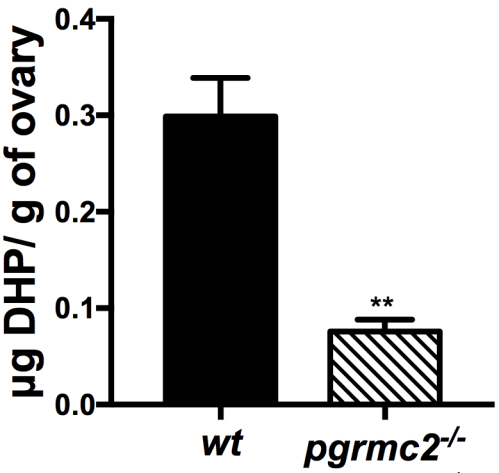


Figure 3. 7 Lower concentration of DHP found in *pgrmc2*$^{-/-}$ ovaries. **$p < 0.01$.

Discussion

Our previous report showed that pgrmc1 regulates female fertility *via* regulation of mPRα and oocyte maturation (Wu et al., 2018). Pgrmc1 did not regulate oocyte maturation directly, but it facilitates plasma localization and expression of mPRα, which in turn indirectly regulates oocyte maturation (Thomas et al., 2014; Thomas et al., 2007; Wu et al., 2018). Oocyte maturation delay was found in *pgrmc2*$^{-/-}$ *in vivo*. Unexpectedly, the oocyte maturation rate (GVBD) *in vitro* was similar in *pgrmc2*$^{-/-}$ compared to those in wildtype zebrafish. Furthermore, the expression of mprs in *pgrmc2*$^{-/-}$ was comparable to those in wildtype individuals. Therefore, the oocyte maturation tardiness found in *pgrmc2*$^{-/-}$ *in vivo*, was not caused by reduced sensitivity of progestin and mPRs expression in the oocytes in the *pgrmc2*$^{-/-}$. This deficiency is likely caused by lower progestin synthesis level found in *pgrmc2*$^{-/-}$ ovaries. We also found that pgrmc2 expressed was relatively high in oocytes and fluctuated during spawning. It is possible that Pgrmc2 has a role in trafficking membrane proteins including mPRα to the oocyte surface, and this role of Pgrmc2 may be compensated by Pgrmc1 in *pgrmc2*$^{-/-}$. Previous studies demonstrated that PGRMC1 binds cytochrome P450(CYP) proteins: CYP51, CYP21A2, CYP21, CYP7A1 and CYP3A4 and alters

the activities of these proteins (Hughes et al., 2007). The conservation of cytochrome b5 heme binding domain among Pgrmc1 and Pgrmc2 suggest Pgrmc2 may also has a role in steroid synthesis (Cahill, 2007). The expressions of several steroidogenesis enzymes were downregulated in our *pgrmc2*$^{-/-}$. CYP11A1 is a mitochondrial enzyme that catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones. HSD3B1 catalyzes the biosynthesis of progesterone from pregnenolone. Reduced expression of cyp11a1 and hsd3b1 and in *pgrmc2*$^{-/-}$ ovaries likely leads to reduced steroid synthesis including progestin, which causes oocyte maturation tardiness in *pgrmc2*$^{-/-}$. The expression level of hsd17b1 is also lower in the *pgrmc2*$^{-/-}$. This enzyme is responsible for the interconversion of estrone (E1) and estradiol (E2), and for the interconversion of androstenedione and testosterone. Only one previous study suggested that PGRMC2 might have a role in steroid synthesis. Albrecht and colleagues showed PGRMC2 was detected in the endoplasmic reticulum (ER) in SKOV-3 cells, and PGRMC2 interacts with both the cytochrome P450 proteins CYP21A2 and CYP3A4 in HEK293T cells (Albrecht et al., 2012). Since CYP21A2 plays an important role in steroidogenesis and CYP3A4 in bile acid synthesis (DeBose-Boyd, 2007), Albrecht et al suggested PGRMC2 might be involved in cholesterol metabolism pathways like bile acid synthesis and steroidogenesis (Albrecht et al., 2012). To our knowledge, this is the first evidence of Pgrmc2 in steroidogenesis in an animal model *in vivo*. However, further studies are required to understand the underlying mechanisms of Pgrmc2 in the regulation of steroid synthesis.

To date, the identities of nongenomic progestin receptors in meiosis resumption and oocyte maturation are still the subject of hot debate. Pgrmc2 is unlikely the membrane progestin receptor that is essential for meiosis resumption and oocyte maturation process, though Pgrmc1 and Pgrmc2 have been suggested to be the membrane progestin receptors (McCallum et al., 2016; Peluso et al.,

2014). This hypothesis is supported by our results, i.e., oocytes from *pgrmc2*$^{-/-}$ has similar sensitivity to progestin as oocytes from wildtype. To our knowledge, there is no study that indicates Pgrmc2 binds progestins with high affinity. In addition, PGRMC2 siRNA treatment does not reduce the binding capacity of spontaneously immortalized rat granulosa cells (SIGCs) to P4 (Peluso et al., 2014). However, studies about Pgrmc2 are very limited, more studies are required to understand the roles of Pgrmc2 in steroid synthesis and oocyte maturation.

Both Pgrmc1 and Pgrmc2 are important for the female fertility. Reduced-fertility phenotypes were found in both *pgrmc1*$^{-/-}$ and *pgrmc2*$^{-/-}$ zebrafish, and also in the PGRMC1 and PGRMC2 conditional knockout mice (Clark et al., 2016; McCallum et al., 2016). Besides the delay of oocyte maturation in Pgrmc mutant zebrafish, a higher percentage of stage I oocytes also were found in *pgrmc1*$^{-/-}$ and *pgrmc2*$^{-/-}$ females than in wildtype, which may suggest a role of Pgrmc2 in early oogenesis by regulating expression of other surface receptors such as Fshr. The Fshr knockout leads to failure of follicle activation with all ovarian follicles arrested at the primary growthprevitellogenic transition in female zebrafish (Zhang et al., 2015). Lower expression of *fshb* and *lhcgr* in *pgrmc2*$^{-/-}$ may also likely contribute to a deficit in follicle growth and a higher percentage of stage I oocytes. This may also contribute to the reduced fertility found in *pgrmc2*$^{-/-}$ females.

In summary, Pgrmc2 has roles in fertility and in steroid synthesis by regulating expressions of steroid synthesizing enzymes. The Pgrmc2 mutation lead to reduced progestin syntheses, which can cause oocyte maturation tardiness *in vivo*. However, further studies are required to elucidate molecular mechanisms underlying the actions of Pgrmc2.

CHAPTER 4: Downregulation of Nuclear Progestin Receptor (Pgr) and Subfertility in Double Knockouts of Progestin Receptor Membrane Component 1 (*pgrmc1*) and *pgrmc2* in Zebrafish

Wu, X.-J., Zhu, Y., 2019. Downregulation of nuclear progestin receptor (Pgr) and subfertility in double knockouts of progestin receptor membrane component 1 (pgrmc1) and pgrmc2 in zebrafish. General and comparative endocrinology, 113275.

Chapter summary

The progestin receptor membrane components (Pgrmcs) contain two paralogs, Pgrmc1 and Pgrmc2. Our previous research into single knockout of Pgrmc1 or Pgrmc2 suggests that Pgrmc1 and Pgrmc2 regulate membrane progestin receptor or steroid synthesis and therefore female fertility in zebrafish. Additional roles of Pgrmcs may not be determined by using single Pgrmc knockouts due to compensatory roles between Pgrmc1 and Pgrmc2. To address this question, single knockout *pgrmc1* (*pgrmc1*$^{-/-}$) was crossed with *pgrmc2* (*pgrmc2*$^{-/-}$) to generate double knockouts for both *pgrmc1* and *pgrmc2* (*pgrmc1/2*$^{-/-}$) in a vertebrate model, zebrafish. In addition to the delayed oocyte maturation and reduced female fertility, significant reduced ovulation was found in double knockout (*pgrmc1/2*$^{-/-}$) *in vivo*, though not detected in either single knockout of Pgrmc (*pgrmc1*$^{-/-}$ or *pgrmc2*$^{-/-}$). Significant down regulation of nuclear progestin receptor (Pgr) protein expression was found only in *pgrmc1/2*$^{-/-}$, which was most likely the cause of reduced ovulation. Lower protein expression of Pgr also resulted in reduced expression of metalloproteinase in *pgrmc1/2*$^{-/-}$. With this study, we have provided new evidence for the physiological functions of Pgrmcs in the regulation of female fertility by regulation of ovulation, likely *via* regulation of Pgr, which affects regulation of metalloproteinase expression and oocyte ovulation.

Introduction

Ovulation is a physiological process where a mature, fertilizable oocyte is released from the surrounding follicular cells. The degradation in extracellular matrix of the oocytes is paramount for follicular rupture to occur. In vertebrates, ovulation is triggered by a luteinizing hormone (Lh) surge from the pituitary, which signals *via* the receptor (Lhcgr) in the granulosa cell. This initial signal then amplified *via* progestin synthesis in preovulatory follicles, which in turn activates nuclear progestin receptor (Pgr). The role of Pgr in ovulation is highly conserved across vertebrate species, as is evidenced by the inability of Pgr knockout female zebrafish, rats, and mice to ovulate (Kubota et al., 2016; Lydon et al., 1995; Zhu et al., 2015). Further studies indicated that Pgr is an upstream regulator important for the increased expression of proteolytic enzymes and therefore promotes the rupture of follicles for releasing mature oocytes during ovulation (Liu et al., 2017; Liu et al., 2018).

As the downstream targets of Pgr, several proteolytic enzymes that may be responsible for follicle rupture during ovulation have been identified (Ogiwara et al., 2005; Takahashi et al., 2013). Several members of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family have been proposed to be important for ovulation. For example, MMPs and ADAMTS family were found to be induced in the ovarian follicles (Peluffo et al., 2011; Piprek et al., 2018; Rosewell et al., 2015). In addition, inhibitors of MMPs using antibodies drastically suppressed *in vitro* ovulation in medaka (Ogiwara et al., 2005). Pharmacologic inhibition of metalloproteases also indicates the importance of these proteinases in ovulation (Butler et al., 1991; Reich et al., 1985). However, since mice that lack metalloproteinases die either in utero or shortly after birth (Carmeliet et al., 1993; Enomoto et al., 2010; Holmbeck et al., 1999; Kelly et al., 2005; Peschon

et al., 1998; Vu and Werb, 2000), the roles played by these proteases in ovulation remains unclear. As in mammals, only two progestin receptor membrane component (Pgrmc) paralogs (Pgrmc1 and Pgrmc2) have been identified in zebrafish. They have been suggested as progestin receptors, or adaptor proteins, which facilitate other receptors to mediate progestin signaling (Peluso et al., 2008a; Thomas, 2008; Zhu et al., 2008). Pgrmcs take part in various physiological and biochemical processes that are important for normal reproduction. For instance, Pgrmc1 is regulated by progesterone and can also affect progesterone metabolism (Rohe et al., 2009). In addition, Pgrmc1 may affect oocyte maturation *via* regulating expression and trafficking of mPRα to localize at cell surface (Thomas et al., 2014; Wu et al., 2018). Furthermore, Pgrmc1 has also been suggested to mediate antiapoptotic and antimitotic actions of progesterone in rat granulosa cells (Peluso et al., 2006). Recent investigations in Pgrmc1 and Pgrmc2 knockout mice have suggested that Pgrmcs are required for normal fertility in females. Conditional ablation of Pgrmc1 results in reduced fertility in female mice, while knocking out Pgrmc2 causes premature reproductive senescence in female mice, possibly due to post-implantation failure (Clark et al., 2016; McCallum et al., 2016). Similar reduced fertility was also observed in *pgrmc1*$^{-/-}$ and *pgrmc2*$^{-/-}$ zebrafish (Wu et al., 2019; Wu et al., 2018). However, whether Pgrmc1 and Pgrmc2 compensate each other is still unknown. In the present study, double knockouts for Pgrmc genes were generated in zebrafish (*Danio rerio*) using CRISPR/Cas9 gene editing technology, characterized phenotypes of these knockouts, and examined the molecular mechanisms underlying the actions of Pgrmc. We found reduced spawning frequencies and number of embryos in double knockouts *pgrmc1/2*$^{-/-}$. In addition, reduced ovulation *in vivo* was observed. Significant reduced Pgr protein expression in *pgrmc1/2*$^{-/-}$ likely caused, at least partially, for attenuated ovulation in *pgrmc1/2*$^{-/-}$.

Materials and methods

Zebrafish

The wildtype zebrafish (*Danio rerio*) strain used in this investigation, the Tübingen strain, was initially obtained from the Zebrafish International Resource Center, then propagated in our lab at East Carolina University. Double knockouts *pgrmc1/2*$^{-/-}$ were generated by crossing of *pgrmc1*$^{-/-}$ with *pgrmc2*$^{-/-}$ (Wu et al., 2019; Wu et al., 2018). Fish were kept under a photoperiod of 14h light and 10h dark (lights on at 09:00, lights off at 23:00), with water temperature around 28.5℃, pH ~7.2, and salinity conductivity ranging from 700 to 1,200 μS in automatically controlled zebrafish rearing systems (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed twice a day and supplied with newly hatched brine shrimp. All the animal care and experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

Spawning and fertility

After zebrafish reached their maturity at ~ 4-months of age, 10 homozygous *pgrmc1/2*$^{-/-}$ female fish were crossed with fertility confirmed wildtype males. Number of offspring was recorded daily for a period of two weeks following a two-week acclimation period. Spawning frequency was defined as the number of times a female produce fertilized embryos in a two-week examination period.

Follicle isolation and quantification of different stage follicles

Oocyte maturation in zebrafish typically occurs prior to the onset of (day) light, while ovulation and spawning occurs within 1 hr following the onset of light. Therefore, adult females (n = 7) from different genotypes (*wt* and *pgrmc1/2*$^{-/-}$) were euthanized at 09:30am, thirty minutes after laboratory lights were turned on, by placing each fish in a lethal dose of MS-222 (300 mg/L

buffered solution) for 10 minutes, then severing the spinal cord and blood supply using IACUC approved procedures. The ovaries of each fish were then immediately dissected out and rinsed in 60% L-15 media (Sigma-Aldrich, St. Louis, MO, USA) containing 15 mM HEPES (pH=7.2). Follicles of various sizes were isolated from the ovaries using fine forceps. Thereafter, ovaries were transferred to a 15-ml centrifuge tube and pipetted up and down to separate the follicles. Then the separated ovaries were transferred to a 90-mm petri dish containing 60% L-15 media. The diameter of each follicle was measured using a stereo microscope (SZX7, Olympus, Japan). The development of follicles was divided into five stages based on morphological criteria and on physiological and biochemical events (Selman et al., 1993; Tyler and Sumpter, 1996): stage I (<140 μm) and II (140-340 μm) previtellogenic follicles; stage III early vitellogenic follicles (340-690 μm); stage IV late vitellogenic follicles (690-730 μm), which is comprised of two stages, immature stage IVa oocytes (before germinal vesicle breakdown, i.e., GVBD) and mature stage IVb (underwent GVBD but haven't yet gone through ovulation); and stage V ovulated follicles (730-750 μm), characterized as ovulated eggs with no follicular cells attached.

RNA isolation and Real-time quantitative PCR

Based on the size standard mentioned above, total RNA was isolated from stage III (21:00), stage IVa (6:00), and stage IVb (08:00) follicles using the RNAzol reagent (Molecular Research Center, Cincinnati, OH, USA) according the manufacturer's protocol. The quantity and quality of the RNA was determined using a Nanodrop 2000 (Thermo Fisher). RNA samples were then reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using SYBR green with C1000 Touch Thermal Cycler (Bio-Rad). PCR efficiency was calculated from the equation of efficiency (EFF) = 10(−1/slope) − 1 and authentic PCR products were confirmed by analyses of melting curve, gel

electrophoresis, and DNA sequencing. PCR data was analyzed using the absolute quantitation method, expressed as copies/μg RNA, and was determined using Ct values of samples and a standard curve from serial known concentrations of plasmids containing different cDNA fragment of target genes. Comparative Ct method was not used in this study because house-keeping-genes vary between different developmental stages of follicles (Liu et al., 2018). The primers used in this study can be found in our previously published work (Liu et al., 2018).

Western blotting

Expression of Pgr in the fully-grown stage IVa immature follicles was confirmed by Western blot analysis using a previously developed polyclonal antibody for Pgr (Hanna and Zhu, 2011). Total protein from 10 stage IVa follicles (06:00) that was collected directly from freshly sacrificed fish was sonicated in 100 μl of 1x SDS sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 100 mM Dithiothreitol) on ice for about 10 short bursts (Sonic Dismembrator, Fisher Scientific). Samples were then immediately boiled for 10 minutes and stored in -20 ℃ freezer until Western blot analysis. 10 μL of each sample was loaded onto 8% SDS PAGE gel and transferred to a nitrocellulose membrane. The membrane was first pre-incubated for 3 hrs with a blocking solution containing 5% BSA (albumin from bovine serum, Sigma A7906) in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4), then with a primary antibody (Pgr, 1:250 dilution; alpha-Tubulin (Sigma, T6074), 1:3000 dilution) in the 1% BSA blocking solution overnight. The following day, the membrane was washed five times for a period of 5 minutes each with 1x TBST, incubated for 2 hr with horseradish peroxidase conjugated secondary antibody (1:5000 dilutions, goat anti-rabbit antibody for Pgr detection or goat anti-mouse antibody for α-Tubulin), and finally washed five times for a period of 5 minutes each with 1x TBST. The membranes were developed using Super Signal West Extended Dura Substrate (Pierce, Rockford,

IL, USA) in a plastic wrap, then visualized using a Fluor Chem 8900 imaging station (Alpha Innotech, San Leandro, CA, USA). Protein size was determined by comparison to a biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA) and a prestained protein ladder (Fermentas, Waltham, MA, USA). Finally, image analyzing software (ImageJ) was used to estimate relative densitometries (Schneider et al., 2012).

Statistical analysis

All the results were presented as mean ± SEM. Significant differences among paired treatment groups was determined using unpaired Student's *t* test and among multiple treatment group using One-way analysis of variance (ANOVA) followed by Turkey's test (GraphPad Prism 7.0a, San Diego, CA, USA). Statistical significance was set at $p<0.05$.

Results

Reduced fertility in *pgrmc1/2*$^{-/-}$ female zebrafish

To evaluate the reproductive capacities in *pgrmc1/2*$^{-/-}$ female zebrafish, mature mutant females (n=10) at 4 months of age were mated with known fertile wildtype males during a minimum 4-week mating study period (two weeks of accommodating period followed by two weeks of quantification period). The fecundity of *pgrmc1/2*$^{-/-}$ female zebrafish, determined as total number of embryos produced over two weeks following a two-week acclimation period, was recorded and compared to those in wildtype crossing (wildtype males crossed with wildtype females) that were treated exactly the same during the same time period. A significantly lower number of offspring was produced by *pgrmc1/2*$^{-/-}$ female zebrafish *(pgrmc1/2*$^{-/-}$, n=10, 828.5 ± 131.3, $p<0.0001$) in comparison to those produced by wildtype females (n=10, 1827 ± 63.2) (Figure 4.1A). *pgrmc1/2*$^{-/-}$ females also spawned with significantly less frequency (n=10, 60.71 ±

8.06%) as compared with wildtype females (n=10, 92.14 ± 1.67%) (Figure 4.1B). In addition, embryos produced daily and embryos produced each time female spawned also were significantly lower in *pgrmc1/2*$^{-/-}$ female zebrafish (Figure 4.1C-1E).

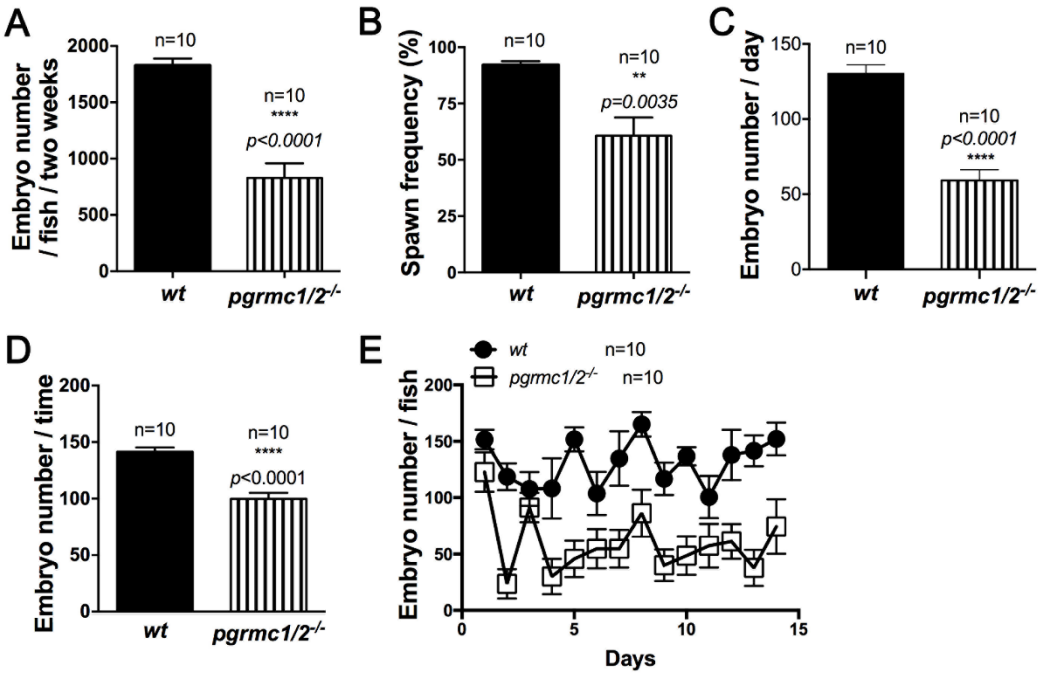


Figure 4.1 Reduced fertility in *pgrmc1/2*$^{-/-}$ homozygous female zebrafish. (A) Mutant female zebrafish produced fewer embryos over a two-week mating period than *wt* females. (B) Mutant females spawned with less frequency than wildtype. (C) *pgrmc1/2*$^{-/-}$ females spawned less embryos per day. (D) *pgrmc1/2*$^{-/-}$ females spawned less embryos each time they spawned. (E) Wildtype females produced more embryos daily than *pgrmc1/2*$^{-/-}$ females. **, $p<0.01$; ****, $p<0.0001$.

Reduced oocyte maturation and ovulation in *pgrmc1/2*$^{-/-}$ *in vivo*

To determine the possible cause for reduced fertility in *pgrmc1/2*$^{-/-}$, ovaries from 4-month-old zebrafish were sampled 30 minutes after lights were turned on (09:30) and the number of different staged follicles were counted. No significant differences were found in the number of early stage, immature follicles (stage II and stage III) in the ovaries from *pgrmc1/2*$^{-/-}$ females in comparison to those from wildtype female fish (Figure 4.2). Typically, fully grown immature follicles (stage IVa) would have already successfully completed processes of oocyte maturation and ovulation, and no stage IVa and stage IVb would be found in the ovaries from wildtype females

after lights were on for half an hour (Figure 4.2). However, a significantly large number of these stage IVa and stage IVb follicles still could be observed in *pgrmc1/2*$^{-/-}$ females (Figure 4.2). These results indicate that oocytes maturation and oocyte ovulation process were affected in *pgrmc1/2*$^{-/-}$.

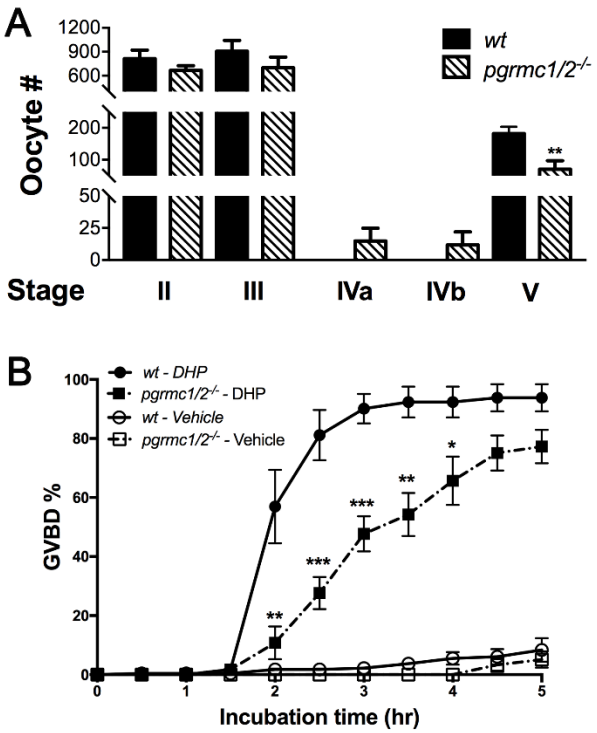


Figure 4.2 Quantification of different stages of oocytes in *wt* and *pgrmc1/2*$^{-/-}$ female ovaries. Significant numbers of fully-grown immature stage IVa follicles and matured but not ovulated stage IVb oocytes were observed in *pgrmc1/2*$^{-/-}$ females 30 minutes after room lights were switched. At the same time point, no stage IVa and stage IVb follicles could be observed in wildtype females. Further, *pgrmc1/2*$^{-/-}$ females had less stage V oocytes. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Pgr was downregulated in *pgrmc1/2*$^{-/-}$

To identify the possible genes underlying the reduced ovulation found in *pgrmc1/2*$^{-/-}$, we examined the expression of Pgr in the Pgrmc mutants as Pgr is a key upstream regulator for ovulation. The RNA expression of *pgr* was significantly lower in *pgrmc1*$^{-/-}$ single mutants in stage III oocytes collected at 21:00, in stage IVa oocytes sampled at 06:00 in *pgrmc1*$^{-/-}$ and *pgrmc1/2*$^{-/-}$, and in stage IVb oocytes sampled at 08:00 in all Pgrmc mutants (Figure 4.3A). The lowest RNA

expression of Pgr was observed in *pgrmc1/2*$^{-/-}$ of stage IVa oocytes (Figure 4.3A). The protein levels of Pgr were significantly reduced in stage IVa follicles (06:00) of *pgrmc1/2*$^{-/-}$, but not in *pgrmc1*$^{-/-}$ or *pgrmc2*$^{-/-}$ single mutant (Figure 4.3B).

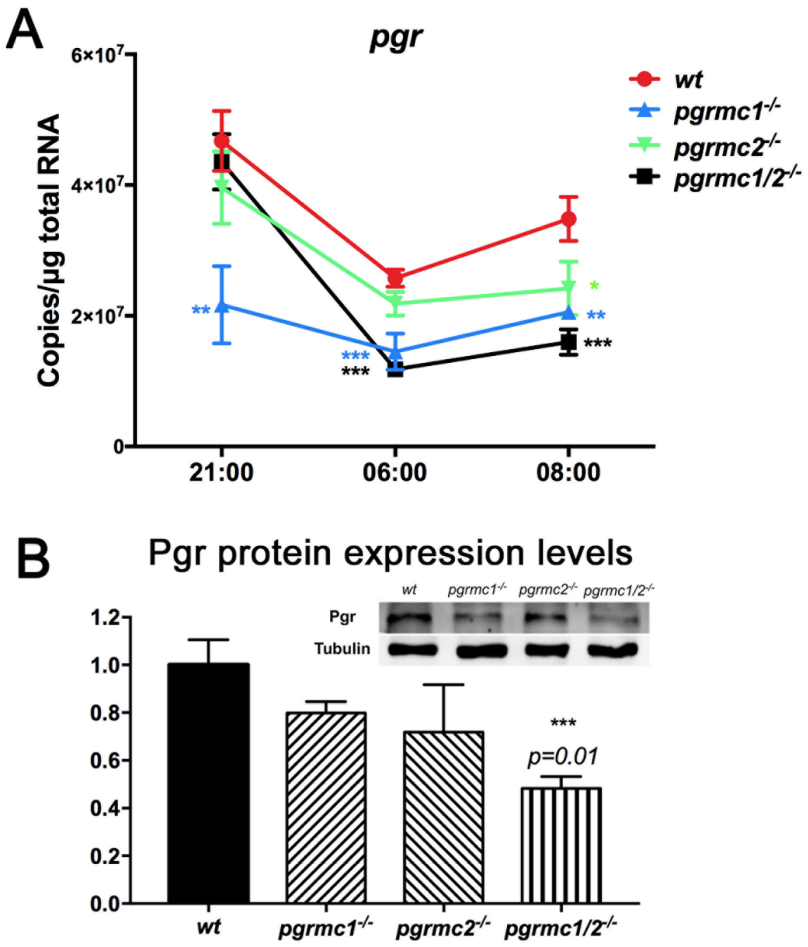


Figure 4.3 Reduced Pgr expression in the follicles of *pgrmc1/2*$^{-/-}$. (A) Expression of *pgr* RNA levels in different stage oocytes from *wt* or Pgrmc mutants over different time points (21:00, stage III; 06:00, stage IVa; 08:00, stage IVb). (B) Reduced expression of Pgr protein in stage IVa follicles (06:00) only in *pgrmc1/2*$^{-/-}$. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Reduced expression of metalloproteinase

Based on the lower expression level of Pgr in *pgrmc1/2*$^{-/-}$, we hypothesized that metalloproteinases required for ovulation will be lower in the *pgrmc1/2*$^{-/-}$, since Pgr regulate metalloproteinases expression during oocyte ovulation. Therefore, we collected different stages of

oocytes and determined expression of six representative metalloproteinases at different critical time points. In the stage III oocytes, *adamts1* RNA expression level was upregulated in *pgrmc2*$^{-/-}$ (Figure 4.4B). Similar, *adamts9* RNA expression in the stage IVa oocytes was high in *pgrmc2*$^{-/-}$ when compared to *wt* (Figure 4.4D). In the stage IVa immature oocytes, *mmp9* were reduced in all three kinds of Pgrmc mutants (Figure 4.4F). In the stage IVb oocytes, *adam8b* was higher expressed in *pgrmc2*$^{-/-}$ than *wt* (Figure 4.4A) and expression of *adamts9* were lower in all Pgrmc mutants (Figure 4.4D). Only in the *pgrmc1/2*$^{-/-}$ stage IVb oocytes, *adamts1*, *adamts8a*, and *mmp2* were lower expressed when compared to *wt* (Figure 4.4B, 4.4C, 4.4E).

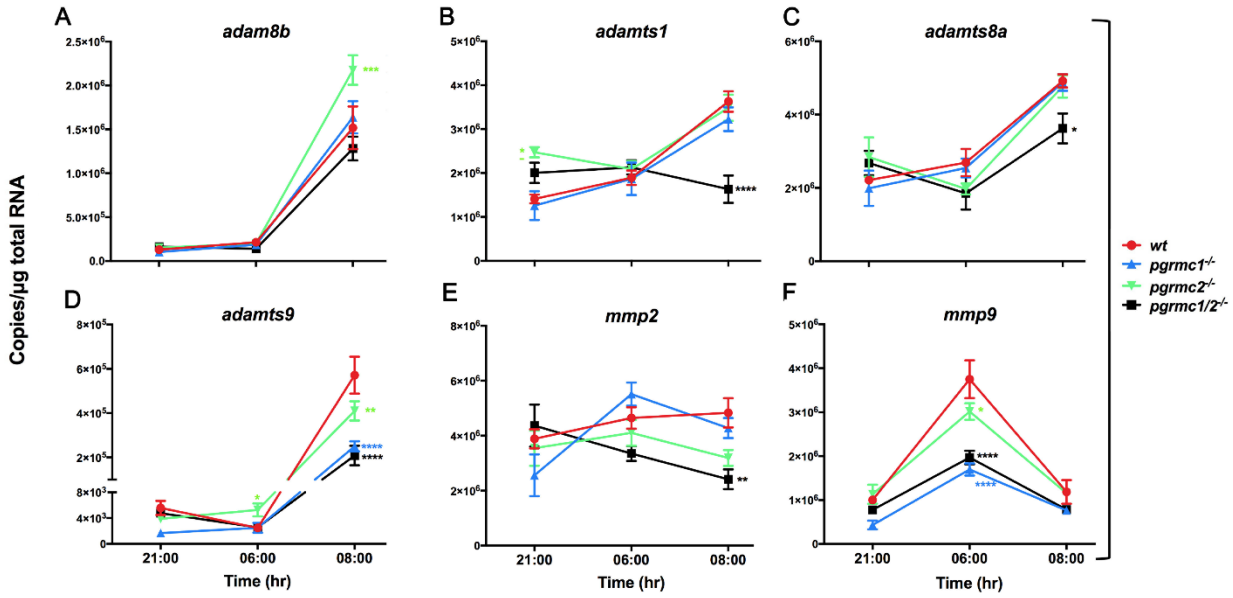


Figure 4. 4 Expression of metallopeptidase in Pgrmc knockouts in comparison to *wt*. Lower *adamts9* expression can be observed in stage IVb oocytes of all genotypes (*pgrmc1*$^{-/-}$, *pgrmc2*$^{-/-}$, and *pgrmc1/2*$^{-/-}$). In addition, expression of *adamts1*, *adamts8a*, and *mmp2* was significantly reduced in stage IVb follicles in *pgrmc1/2*$^{-/-}$. Asterisks indicate a significant difference of transcripts compared to *wt* at the same time point. adam8b, a distintegrin and metalloproteinase domain 8b; adamts1, a disintegrin and metalloproteinase with thrombospondin type 1 motif 1; adamts8a; adamts9; mmp2, matrix metalloproteinase 2; and mmp9, matrix metalloproteinase 9. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. 21:00, stage III; 06:00, stage IVa; 08:00, stage IVb.

Discussion

Pgrmc1 and Pgrmc2 have been suggested as important adaptors in mediating progesterone signaling and steroid synthesis, but their functions *in vivo* have not been well established. To our knowledge, this is the first evidence that Pgrmc1 and Pgrmc2 play a role in the regulation of Pgr and oocyte ovulation. We found reduced oocyte ovulation in *pgrmc1/2*$^{-/-}$ females *in vivo* but not in *pgrmc1*$^{-/-}$ or *pgrmc2*$^{-/-}$ (Wu et al., 2019; Wu et al., 2018). One main cause for reduced oocyte ovulation in *pgrmc1/2*$^{-/-}$ fish is likely due to dramatically reduced protein expression of Pgr, which is the essential regulator of oocyte ovulation. Lower protein expression of Pgr subsequently caused abnormally low metalloproteinase expression and resulted in reduced ovulation. Our results indicate an interaction between two different progestin receptor families, which operate with each other to regulate ovulation.

Low Pgr causes reduced ovulation

It is well established that Pgr is essential for ovulation across species. In agreement with other studies, *pgr* also increases prior to ovulation in zebrafish (Bayaa et al., 2000; Hanna and Zhu, 2011; Hild-Petito et al., 1988; Liu et al., 2018; Park and Mayo, 1991; Press and Greene, 1988; Richards and Ascoli, 2018). Selective PGR antagonist RU486 or CDB-2914 can reduce the number of ovulated oocytes in mice (Loutradis et al., 1991; Palanisamy et al., 2006). In addition, the oocytes remain trapped within follicles in Pgr$^{-/-}$ mice and zebrafish because pre-ovulatory follicles are unable to undergo follicle-wall degradation (Lydon et al., 1995; Zhu et al., 2015). Our study demonstrated that low Pgr protein expression in *pgrmc1/2*$^{-/-}$ zebrafish also resulted in ovulation reduction *in vivo*, supporting the notion that Pgr is a pivotal factor for oocyte ovulation.

Pgrmc1 and Pgrmc2 affect gene expression of Pgr

A previous study in mice showed ablation of Pgrmc1/2 did not alter the RNA expression of the Pgr (Clark et al., 2016), but immunohistochemistry results showed less staining in the Pgrmc1/2$^{d/d}$ uterine. Similarly, we also detected low Pgr transcription and protein level in *pgrmc1/2*$^{-/-}$ zebrafish. The relationship between these two progesterone receptor families is still unclear. Previous research suggests a compensatory mechanism between Pgrmc1 and Pgr since higher levels of Pgrmc1 have been found in the brains of Pgr-KO female mice than in their wild-type littermates (Krebs et al., 2000). Also, activation of Pgr can represses expression of Pgrmc1 during lordosis facilitation (Krebs et al., 2000). In our results, we found a new relationship between Pgrmcs and Pgr; and Pgrmcs are important for normal expression of Pgr. But how Pgrmc1 and Pgrmc2 regulates the expression of Pgr remains unknown and needs further investigation. In our previous work, we showed Pgrmc1 and Pgrmc2 to be highly expressed in the follicular cells and denuded oocytes (Wu et al., 2019; Wu et al., 2018). In interphase cells, Pgrmc1 was distributed throughout the cell including the nucleus (Peluso et al., 2014). But whether Pgrmcs can bind to transcription factors and directly affect Pgr gene expression remains unclear. PGRMC1 interacts with epidermal growth factor receptor (EGFR) (Ahmed et al., 2010; Aizen and Thomas, 2015; Kabe et al., 2016) and membrane progesterone receptor α (mPRα) (Aizen et al., 2018; Thomas et al., 2014). The downstream signaling of EGFR and mPRα may result in gene expression differences of Pgr. Pgrmc1 and Pgrmc2 also co-localized to cytoplasm (Peluso et al., 2014), indicating a close relationship between Pgrmc1 and Pgrmc2. Pgrmc1 and Pgrm2 may have similar, but not identical, functions because double knockout (*pgrmc1/2*$^{-/-}$) female zebrafish had a more severe reproductive phenotype than those found in *pgrmc1*$^{-/-}$ or *pgrmc2*$^{-/-}$ single knockout female

zebrafish, respectively. This unknown function from Pgrmcs may explain why oocyte ovulation delay and Pgr protein decrease only happens in *pgrmc1/2*$^{-/-}$ but not single mutants.

Lower proteinase expression causes ovulation delay

In late stage follicles, such as stage IVa and stage IVb, the follicular cells surrounding the oocyte synthesize a variety of factors to induce ovulation, including kinds of metalloproteinases. This process is under the control of Pgr (Liu et al., 2017). The involvement of several metalloproteinases, including members of MMP (matrix metalloproteinase), ADAM (A Disintegrin And Metalloproteinases), and ADAMTS families, has been examined (Brown et al., 2010; Peluffo et al., 2011; Robker et al., 2000; Sriraman et al., 2008). In our *pgrmc1/2*$^{-/-}$ follicles, we observed downregulation of kinds of metalloproteinases. Adamts1 has been identified as an important metalloproteinase exert functions in oocyte ovulation. ADAMTS1 cleaves the surrounding versican-rich matrix of oocytes and allows the release of the oocyte (Brown et al., 2010; Robker et al., 2000). In Adamts1$^{-/-}$ mice, both ovulation and subsequent fertilization were severely impaired as a result of the versican persistence (Brown et al., 2010; Mittaz et al., 2004; Shozu et al., 2005). Adamts1 transcripts increased in mice granulosa cells of periovulatory follicles, with this high expression being induced by hCG and progesterone. But this induction is impaired in *Pgr*$^{-/-}$ ovaries, indicating that Adamts1 acts downstream of Pgr (Robker et al., 2000). In our Pgrmc mutant lines, low *adamts1* expression can only be seen in the stage IVb oocytes of the *pgrmc1/2*$^{-/-}$. Besides Adamts1, Adamts9 also plays an important role in oocyte ovulation. Due to embryonic lethality in Adamts9$^{-/-}$ (Dubail et al., 2014; Enomoto et al., 2010), the function of this protein during ovulation is unknown. The *adamts9*$^{-/-}$ zebrafish can survive and grow to adulthood, but the ovaries are not well developed in mutants (Carter et al., 2019). However, Adamts9 expression is induced by LH and hCG in mature follicles in zebrafish, monkeys, and humans

during early ovulation (Liu et al., 2018; Peluffo et al., 2011; Rosewell et al., 2015). Adamts9 is specifically expressed in follicular cells but not in the oocytes and its expression increase significantly prior to ovulation (Liu et al., 2018). In addition, the expression of *adamts9* is significantly downregulated in the follicular cells of anovulatory *pgr*$^{-/-}$ zebrafish (Liu et al., 2018). The proteoglycans aggrecan and versican are known substrates of Adamts9. Therefore, Adamts9 is important for ovulation. Our results demonstrated that *adamts9* expression reduced in *pgrcm1*$^{-/-}$, *pgrmc2*$^{-/-}$, and *pgrmc1/2*$^{-/-}$ when compared to *wt*. It is plausible that reduced ovulation in *pgrmc1/2*$^{-/-}$ might be due to lower level of *adamts9* resulting from the lower levels of Pgr. MMPs may also play critical roles in ovulation through remodeling extracellular matrix (Cooke et al., 1999). In rhesus monkies, granulosa cells exposed to LH *in vitro* showed elevated RNA levels of MMP9 (Duffy and Stouffer, 2003). MMP9 also plays a critical role in LH-induced steroidogenesis in mouse granulosa cells during ovulation (Light and Hammes, 2015). In our Pgrmc mutant lines, transcription of *mmp9* showed a peak in stage IVa oocytes and drop in stage IVb oocytes. This *mmp9* peak is prior to that of Adams and Adamts expression, indicating a different role Mmp9 play in oocyte ovulation. Compared to *wt*, both *mmp2* and *mmp9* are lower in the *pgrmc1/2*$^{-/-}$. Overall, the RNA expressions of *adamts9* and *mmp9* were also low in the *pgrmc1*$^{-/-}$ and *pgrmc2*$^{-/-}$, but we did not observe ovulation delay in single-knockout Pgrmc mutants *in vivo*. Other proteinases like *adamts1*, *adamts8a*, and *mmp2* may contribute to abnormal ovulation in *pgrmc1/2*$^{-/-}$. These proteinases break down different extracellular matrix of oocytes.

Oocyte maturation delay in *pgrmc1/2*$^{-/-}$

In *pgrmc1/2*$^{-/-}$, we also found some stage IVa oocytes after lights were on for 30 mins; and GVBD experiments also show reduced oocyte maturation *in vitro*. Our previous studies show the different roles that Pgrmc1 and Pgrmc2 play in oocyte maturation. Pgrmc1 promotes oocyte

maturation through increased plasma localization and expression of mPRα (Thomas et al., 2014; Thomas et al., 2007; Wu et al., 2018). Further, oocyte maturation reduced in *pgrmc1*$^{-/-}$ *in vivo* and *in vitro* (Wu et al., 2018). In contrast, the maturation rate of fully-grown immature oocytes from *pgrmc2*$^{-/-}$ was similar to those found in wildtype zebrafish *in vitro*. Lower progestin synthesis enzymes were observed in *pgrmc2*$^{-/-}$, which may cause reduced oocyte maturation delay (Wu et al., 2019). Therefore, it is likely that the oocyte maturation delay found in *pgrmc1/2*$^{-/-}$ *in vivo* and *in vitro* is caused by both Pgrmc1 and Pgrmc2.

This is the first time it has been shown that both Pgrmc1 and Pgrmc2 are required for the regulation of Pgr signaling and function in ovulation. Losing both Pgrmcs ultimately leads to transcriptional and translational changes of Pgr. Information obtained from the present study will contribute to our understanding of the interaction between different progesterone receptors. Further research is needed to understand why Pgrmc1 and Pgrmc2 can affect the gene expression of Pgr.

CHAPTER 5: Impaired Oocyte Maturation and Ovulation in Membrane Progestin Receptor (mPR) Knockouts in Zebrafish

Chapter summary

Accumulating evidence suggest that membrane progestin receptor α (mPRα) is the membrane receptor mediating nongenomic progestin signaling that induces oocyte maturation in teleost. However, the involvement of other members of mPR family in oocyte maturation is still unclear. In this study, we found impaired oocyte maturation in zebrafish lacking mPRα1, mPRα2, mPRβ, or mPRγ2. In contrast, no difference was observed in oocyte maturation in the single knockout of mPRγ1, mPRδ, or mPRε. To study possible redundant functions of different mPRs in oocyte maturation, we generated a zebrafish line lacking all seven kinds of mPRs (*mprs*$^{-/-}$). We found oocyte maturation was further impaired in *mprs*$^{-/-}$. In addition, oocyte ovulation delay was observed in *mprs*$^{-/-}$ females, which was associated with low levels of nuclear progestin receptor (Pgr), a key regulator for ovulation. We also found reduced fertility in *mprs*$^{-/-}$ female zebrafish. Furthermore, eggs spawned by *mprs*$^{-/-}$ females were of poor quality.

Introduction

Meiosis resumption, i.e. final oocyte maturation is trigged by binding and signaling of progestin to its membrane receptor located at surface of oocytes in fish and amphibians (Nagahama and Yamashita, 2008; Thomas, 2012; Zhu et al., 2008). Membrane progestin receptor α (mPRα) has been suggested to mediate this process (Thomas, 2008; Zhu et al., 2008). The mPRα is a G protein-coupled receptor (GPCR)-like protein that has a high affinity for progestin and is capable of signaling like GPCRs (Pace and Thomas, 2005; Thomas et al., 2007; Zhu et al., 2003a; Zhu et al., 2003b). The mPRα belongs to a progestin and adipoQ receptor superfamily (PAQR) (Tang et al., 2005; Thomas, 2012; Thomas et al., 2007). Five mPR paralogues (mPRα (Paqr7), mPRβ (Paqr8), mPRγ (Paqr5), mPRδ (Paqr6), and mPRε (Paqr9)) have been identified in this family in

all vertebrates. Zebrafish has two additional mPR paralogs (mPRα2 (Paqr7b), mPRγ2 (Paqr5b)), likely due to teleost specific genome duplication (Glasauer and Neuhauss, 2014). Studies conducted so far were focused on the roles and signaling of mPRα or mPRα2. These studies suggest mPRα mediates rapid nongenomic signaling of progestin to induce oocyte maturation (Aizen et al., 2018; Hanna and Zhu, 2011; Thomas, 2008; Zhu et al., 2008). However, the involvement of other members of mPRs are still unclear.

In addition, progestins also signal through nuclear progestin receptor (Pgr) and progesterone receptor membrane components (Pgrmcs) regulating physiological processes such as oogenesis and ovulation (Kubota et al., 2016; Lydon et al., 1995; Wu et al., 2019; Wu and Zhu, 2019; Wu et al., 2018; Zhu et al., 2015). Limited studies have suggested these progestin receptors also regulate physiological processes by interacting and regulating each other (Aizen et al., 2018; Thomas et al., 2014; Wu and Zhu, 2019). Previous studies suggested Pgrmc1 regulates mPRα expression and transport mPRα to cell surface in oocytes (Aizen et al., 2018; Wu et al., 2018). In addition, activation of mPRs leads to transactivation of PGR-B in human myometrium cells (Karteris et al., 2006). These results suggest cross talks between different families of progestin receptors.

This study attempts to fill a knowledge gap by generating mPR mutants for all known mPR paralogues to investigate their roles *in vivo*. We also generated a total mutant line that lacking all seven mPR paralogues in zebrafish, and found these mPRs are critical for oocyte maturation, ovulation and fertility. Furthermore, we observed lacking mPRs also lead to poor-quality eggs in zebrafish.

Materials and methods

Animals

The zebrafish (*Danio rerio*) strain used in this investigation, the Tübingen strain, was initially obtained from the Zebrafish International Resource Center, then propagated in our lab at East Carolina University following previously published guidelines (see (Zhu et al., 2015). All the animal care and use protocols were approved by Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

TALEN assembly and *in vitro* synthesis of TALEN mRNAs

We designed and assembled TALEN molecules using the unit assembly method detailed in Huang et al. (Huang et al., 2011). Using mPRα2 (Paqr7b) as an example (Supplemental Fig. 2), candidate TALEN target sites were identified using the following parameters: (1) nucleotide T was at position 0; (2) length of the spacer and nucleotides that bound to forward or reverse TALEN proteins were between 16 and 22 bp; and (3) a restriction endonuclease site was identified near the center of the spacer for convenient mutation detection and mutation rate estimation. The target was selected near the beginning of the coding sequence (Supplemental Fig. 2; forward target site sequence: ACTGTGATGGTGAGCG, reverse target site sequence: TATGTGTGACTCACGG, and spacer sequence with a NcoI restriction enzyme site was in bold). All assembled TALEN vectors were confirmed using Sanger sequencing.

These assembled TALEN vectors were linearized with *Not* I, gel extracted, and purified using the QIAquick gel extraction kit according to manufacturer's specifications (Qiagen, Germantown, MD), and mRNAs were transcribed using SP6 mMACHINE kit (Ambion, Austin, TX). The transcribed mRNAs were stored at -80°C until use. Immediately prior to microinjection, mRNA was diluted into workable concentrations (100 ng/μl) with nuclease-free water, and mixed

with an equal volume of 0.5% phenol red solution (Sigma P0290, Sigma-Aldrich, St. Louis, MO).

CRISPR/Cas9 target design and preparation of Cas9 RNA and sgRNAs

Using mPRα1 (Paqr7a) as an example, we identified 5'GG-(N_{18})-NGG3' target sequences in exon 1 of mPRα1 (GGCTCTGGTATAGTAGTTACGGG) (Supplemental Fig. 1). Syntheses of Cas9 RNA and single guide RNAs (sgRNAs) were based on a protocol from Chen's lab (Jao et al., 2013). For Cas9 transcripts (nls-zCas9-nls RNA), a template plasmid (pCS2-nls-zCas9-nls) was linearized by NotI digestion, then purified using a QIAprep column (Qiagen, Germantown, MD). Capped Cas9 mRNA (nls-zCas9-nls) was synthesized using mMESSAGE mMACHINE SP6 kit (Fisher Scientific, Hampton, NH) and purified using RNeasy Mini kit (Qiagen). For sgRNAs, template plasmids were linearized by BamHI digestion and purified using a QIAprep column. The gRNAs were generated by *in vitro* transcription using MEGAshortscript T7 kit (Fisher Scientific). The size and quality of the resulting gRNA was confirmed by electrophoresis using a 2% (wt/vol) formaldehyde agarose gel.

Establishment of zebrafish mutant lines (*mprs*$^{-/-}$)

Screening and generation of mutant zebrafish lines followed protocols established previously (Zhu et al., 2015). To generate a founder population (F0), fertilized eggs were collected within 5 minutes of natural spawning between wildtype fish from their crossing tanks, which were set up the previous night. Microinjection was performed on newly fertilized zebrafish embryos at either the one or two-cell stages. Approximately 1 nl of 100 ng/µl of sgRNA and 150 ng/µl of Cas9 mRNA (or 1 nl of 100 ng/ul of TALEN transcripts) were co-injected into the embryos using a glass microcapillary pipette attached to a micromanipulator under a stereomicroscope (Leica MZ6, Wetzlar, Germany). The injection was driven by compressed N2 gas, under the control of a PV820 Pneumatic PicPump (World Precision Instrument, Sarasota, FL). For comparison, and to

estimate mutagenesis efficiency, embryos without microinjection were designated as wildtype and used as controls. A pool of genomic DNA was extracted from 30 well-developed wildtype or CRISPR/Cas9-gRNA-microinjected (TALEN microinjected) embryos two days post-fertilization (dpf) using a HotSHOT method (Meeker et al., 2007). Mutation rates were estimated by comparing band intensities of digested PCR products to intensities of undigested PCR products using T7 endonucleases I assay. The PCR products were cleaned through a Qiagen column, cloned into a TA cloning vector (Chen et al., 2009), and potential mutant clones were selected for DNA sequencing analysis to confirm the presence of a frame-shifting mutation (Zhu et al., 2015).

To identify germline-transmitted mutations, remaining F0 founder embryos were raised to adulthood and outcrossed with wildtype fish. Genomic DNA from each cross was extracted from 30 randomly selected individuals, and they were pooled as F1 embryos, and the status of the target site was analyzed *via* PCR amplification, T7 Endonucleases I assay, and DNA sequencing as described above. The remaining F1 embryos with identified frame shifted mutations were raised to adulthood and were genotyped individually. Genomic DNA was extracted from part of the caudal fin of adult fish in a 50 μl hot alkaline solution and analyzed as above. Heterozygous F1 adults carrying the same frameshift mutant allele were crossed with each other, which yielded wildtype, heterozygous, and homozygous F2 fish that were further characterized genetically and physiologically. The total knockout for mPRs was obtained by crossing different mutant lines.

Germinal vesicle breakdown (GVBD) assay

To determine the sensitivities of oocytes to a maturation inducing steroid, 17α,20β-dihydroxy-progesterone (DHP), oocytes were isolated and incubated with DHP (Hanna and Zhu, 2011; Pang and Thomas, 2009). Gravid female zebrafish were euthanized humanely, following which the ovaries were dissected out then washed several times in 60% Leibovitz L-15 medium

(Sigma-Aldrich), ensuring that the individual oocytes were carefully prepared without damaging the surrounding follicular cell layers per previously established protocols (Hanna and Zhu, 2011; Liu et al., 2017). Fully-grown immature oocytes of the same size (diameter of 550-650 μm) were selected and randomly distributed into the wells of a 24-well plate (~30 oocytes/1 ml medium/well), then treated with DHP dissolved in ethanol. Final concentrations of DHP and ethanol in the incubation medium are 5 nM and 0.1%, respectively. As controls, pure ethanol was added into control wells containing a same number of oocytes collected at the same time from the same group of individual fish, and the rates of GVBD were also recorded. Incubation continued for up to 5 hours, with GVBDs being scored each half hour during the incubation period. All experiments were repeated five times to confirm the results.

Separation of oocytes according to their size and developmental stages

Oocyte maturation (~6:00) and ovulation (~8:00) in zebrafish typically occur prior to the onset of (day) light, while spawning occurs within 1 h following the onset of light. Adult females (n = 7) from each mutant genotype were euthanized at 9:30 am, thirty minutes after laboratory lights were turned on, by placing each fish in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes, then severing the spinal cord and blood supply using IACUC approved procedures. Oocyte maturation and ovulation in zebrafish typically occurs sequentially prior to the onset of lights, while spawning occur within 1 hr following the onset of lights (Liu et al., 2018). The ovaries of each fish were then immediately dissected out and rinsed in 60% L-15 media (Sigma-Aldrich) containing 15mM HEPES (pH=7.2). Oocytes of various sizes were isolated from the ovaries using fine forceps. The diameter of each oocyte was measured with a stereo microscope (SZX7, Olympus, Japan), and recorded. The developmental stages of oocytes were divided into five stages based on morphological criteria (Selman et al., 1993) with slight modification: stage I (<140 μm) and II

(140-340 μm) previtellogenic oocytes; stage III early vitellogenic oocytes (340-690 μm); stage IV late vitellogenic oocytes (690-730 μm) that are further divided into IVa and IVb two stages, IVa is maturational competent fully grown immature oocytes (IVa), IVb is matured oocytes that underwent oocyte maturation but haven't yet gone through ovulation (IVb); and stage V ovulated oocytes (730-750 μm), i.e. ovulated eggs with no follicular cells attached.

RNA isolation and real-time quantitative PCR

Based on the size standard mentioned above, total RNA was isolated from stage III (21:00), stage IVa (6:00), and stage IVb (08:00) oocytes using the RNAzol reagent (Molecular Research Center, Cincinnati, OH) according to a modified protocol (Liu et al., 2017), and reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). The amount and purity of the RNA was determined using a Nanodrop 2000 (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using the SYBR green with C1000 Touch Thermal Cycler (Bio-Rad). PCR efficiency was calculated from the equation of efficiency (EFF) = 10(−1/slope) − 1 and authentic PCR products were confirmed by analyses of gel electrophoresis, DNA sequencing, and the melting curve. Real-time PCR data was analyzed using the absolute quantitation method, expressed as copies/μg RNA, and was determined using Ct values of samples and a standard curve from serial known concentrations of plasmids containing different cDNA fragment of target genes. Primers sequences can be found in our previous studies (Liu et al., 2018; Wu and Zhu, 2019).

Western blotting

Expression of nuclear progestin receptor (Pgr) in the fully-grown stage IVa immature oocytes was determined by the Western blot using a previously developed polyclonal antibody for Pgr (Hanna et al., 2010). In brief, stage IVa oocytes were collected from adult zebrafish following protocols listed previously (Hanna & Zhu, 2011; Liu et al., 2017). The total protein from ten stage

IVa oocytes, collected directly from freshly sacrificed fish, was sonicated in 100 µl of 1x SDS sample buffer (62.5mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 100mM Dithiothreitol) on ice for about 10 short bursts (Sonic Dismembrator, Fisher Scientific). Samples were then immediately boiled for 10 minutes and stored in -20 ℃ freezer until the start of Western blot analysis. 10 µL of each sample was loaded onto 8% SDS PAGE gel and transferred to a nitrocellulose membrane. The membrane was first pre-incubated for 3 hours with a blocking solution containing 5% BSA (Albumin from bovine serum, Sigma A7906) in TBST (50mM Tris, 100mM NaCl, 0.1% Tween 20, pH 7.4), then with a primary antibody (Pgr, 1:250 dilution; α-Tubulin (Sigma, T6074), 1:3000 dilution) in the 1% BSA blocking solution overnight. The following day, the membrane was washed five times for a period of 5 minutes each with 1x TBST, incubated for 2 hours with horseradish peroxidase conjugated secondary antibody (1:3000 dilutions, goat anti-rabbit antibody for Pgr detection or goat anti-mouse antibody for α-Tubulin), and finally washed five times for a period of 5 minutes each with 1x TBST. The membranes were developed using Super Signal West Extended Dura Substrate (Pierce, Rockford, IL) in plastic wraps, then visualized using a Fluor Chem 8900 imaging station (Alpha Innotech, San Leandro, CA). Protein size was determined by comparison between a biotinylated protein ladder (Cell Signaling Technology, Danvers, MA) and a pre-stained protein ladder (Fermentas, Waltham, MA). Finally, ImageJ was used to estimate relative densitometries (Schneider et al., 2012).

Spawning and fertility

After all zebrafish lines reached their maturity at ~ 4-month-old, ten homozygous mutant female fish were crossed with fertility confirmed wildtype males. Production of offspring for each genotype was recorded daily for a two-week period after a 14-day acclimatization period. Gonadosomatic index (GSI) = (weight of ovary/weight of body)*100%.

Chorion and yolk width measurement

Embryos at 30% epiboly stage were selected randomly and the length between two tips of cells was recorded as the width of yolk, and length of widest part of the chorion as width of chorion. In total, 7 individual pairs of *wt* or *mprs*$^{-/-}$ (30 embryos for each individual) were recorded.

Environmental scanning electron microscope (ESEM)

To determine the submicroscopic structure difference on the surfaces of embryos, 16 to 32-cell stage (approximately 2 hours post fertilization) embryos were fixed in 4% paraformaldehyde overnight. Samples were then dechorionated and scanned by ESEM.

Histological examination and percentage of follicles at different stages

Three females from each genotype were anesthetized by submersion in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes, then severed the spinal cord and blood supply using procedures approved by the IACUC at 8 am. Ovaries were quickly removed and histological examination was performed following protocols described previously (Sullivan-Brown et al., 2011). Briefly, fresh ovaries were fixed overnight in 4% PFA, processed and embedded in JB-4 resin, cut into 5 μm sections, and stained with hematoxylin and eosin. The stages of oocytes were divided according to size and morphological characteristics described in a previous section. Each stage of oocytes was expressed as a percent of the total number of oocytes from both ovaries of each female used.

Statistical analysis

All the results were analyzed using GraphPad Prism 7.0a (San Diego, CA, US) and presented as mean ± SEM. Significant differences among paired treatment groups were determined using Student's *t*-test. GVBD rates at different time points were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical significance was

set at $p < 0.05$.

Results

Impaired oocyte maturation in individual mPR mutants

We generated mutant lines for each mPR paralogue, and conducted germinal vesicle breakdown (GVBD) assays in each of mPR mutant. Fully-grown immature stage IVa oocytes from *mprα1*$^{-/-}$ (*paqr7a*$^{-/-}$), *mprα2*$^{-/-}$ (*paqr7b*$^{-/-}$), *mprβ*$^{-/-}$ (*paqr8*$^{-/-}$), and *mprγ2*$^{-/-}$ (*paqr5b*$^{-/-}$) showed impaired oocyte maturation in response to DHP stimulation, but not those from *mprγ1*$^{-/-}$ (*paqr5a*$^{-/-}$), *mprδ*$^{-/-}$ (*paqr6*$^{-/-}$), and *mprε*$^{-/-}$ (*paqr9*$^{-/-}$) (Figure 5.1). Among single mutants, *mprα2*$^{-/-}$ (*paqr7b*$^{-/-}$) oocytes exhibited the most significant reduced oocyte maturation (Figure 5.1B). In all single mutants, spontaneous oocyte maturation still could happen at low rates when treated with ethanol only (Figure 5.1).

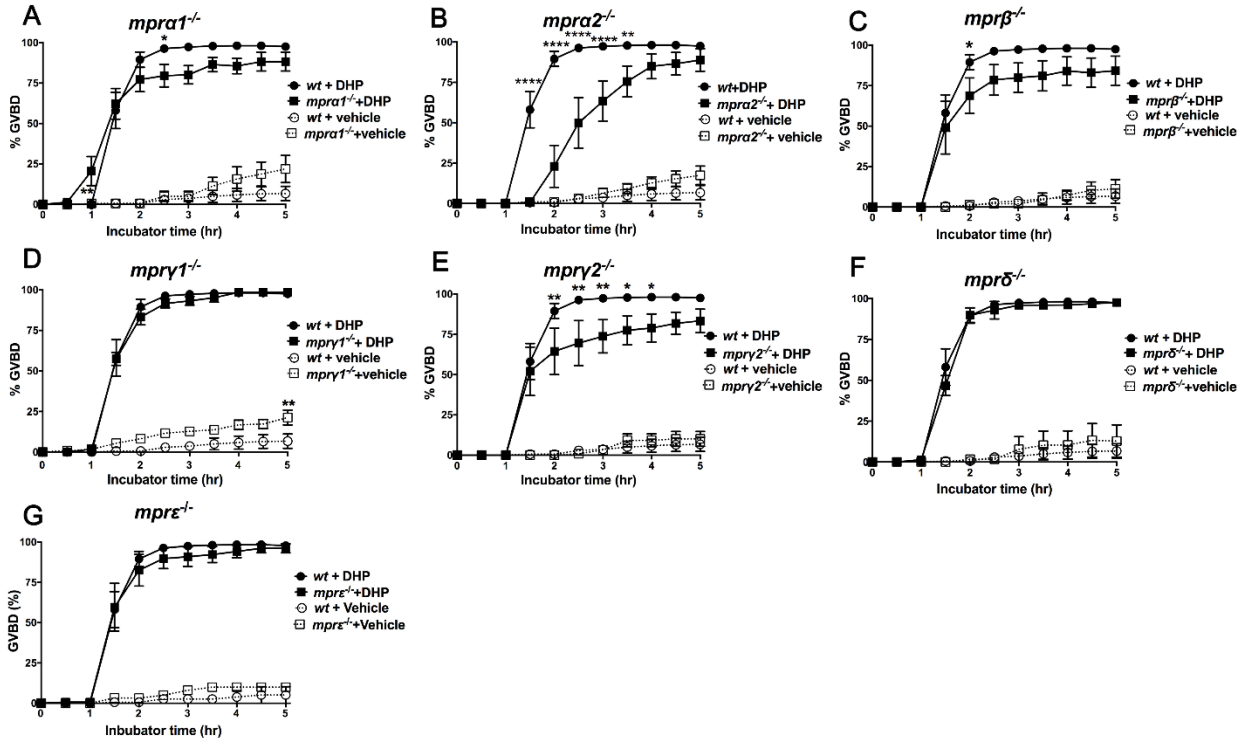


Figure 5. 1 Inhibition of germinal vesicle breakdown (GVBD, i.e. final oocyte maturation) in stage IVa fully-grown immature oocytes in the knockouts of individual membrane progestin receptor (mPR) paralogues in comparison to those from wildtype (*wt*). Stage IVa immature oocytes were exposed to 5 nM exogenous 17α,20β-dihydroxy-progesterone (DHP) or vehicle (0.1% ethanol) and incubated at room temperature for up to 5 hours, rate of GVBD were determined every 30 minutes. (A) *mprα1*$^{-/-}$ (*paqr7a*$^{-/-}$); (B) *mprα2*$^{-/-}$ (*paqr7b*$^{-/-}$); (C) *mprβ*$^{-/-}$ (*paqr8*$^{-/-}$); (D) *mprγ1*$^{-/-}$ (*paqr5a*$^{-/-}$); (E) *mprγ2*$^{-/-}$ (*paqr5b*$^{-/-}$); (F) *mprδ*$^{-/-}$ (*paqr6*$^{-/-}$); (G) *mprε*$^{-/-}$ (*paqr9*$^{-/-}$). The results shown as average (mean ± SEM) of data from five representative experiments. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

Impaired oocyte maturation in a mutant lacking all seven mPR paralogs (*mprs*$^{-/-}$)

To determine possible gene redundancy among mPR paralogs, a zebrafish line lacking all 7 mPRs (*mprs*$^{-/-}$) was generated. Immature stage IVa oocytes can be found in 4 out of 9 *mprs*$^{-/-}$ females after lights were on; in contrast, only 1 out of 9 *wt* female possessed immature stage IVa oocytes at same time point. The number of stage IVa oocytes was higher in *mprs*$^{-/-}$ females than *wt*, but was not significant. Abnormal higher frequency of stage IVa oocytes in *mprs*$^{-/-}$ females indicates impaired oocyte maturation *in vivo* (Figure. 5.2A). This impaired oocyte maturation *in vivo* could be due to less sensitivity of fully-grown immature oocytes to progestin in *mprs*$^{-/-}$. In GVBD assays, fully-grown immature oocytes from *mprs*$^{-/-}$ exhibited significantly reduced maturation over a 5-hour treatment (Figure 5.2B-5.2D). The reduction of GVBD in *mprs*$^{-/-}$ was significantly lower than those observed in single mPR mutants including *mprα2*$^{-/-}$ (Supplemental Fig. 5.8). Even being treated with DHP for 5 hours, only ~70% immature oocytes from *mprs*$^{-/-}$ became matured. In contrast, around 100% of wildtype oocytes matured after treated with DHP for 3 hours. Interesting, around 20% of oocytes from wildtype underwent maturation spontaneously after treated with vehicle (ethanol) for 5 hours. In contrast, none of the oocytes from *mprs*$^{-/-}$ matured spontaneously when treated with vehicle only (Figure 5.2B).

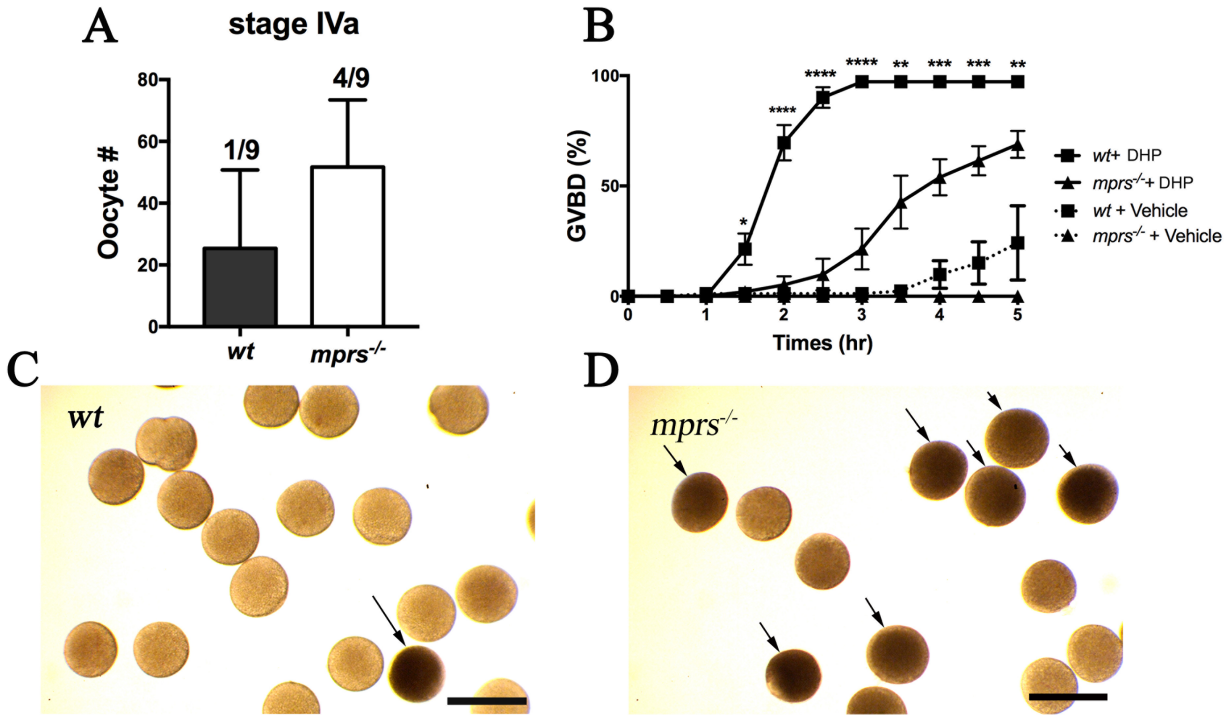


Figure 5. 2 Further reduction of progestin sensitivities in stage IVa fully-grown immature oocytes with all seven membrane progestin receptor paralogues mutated (*mprs*$^{-/-}$). (A) Increased number of females with stage IVa oocytes found in ovaries from *mprs*$^{-/-}$ *in vivo*. Ovaries were collected at 0.5 hours following lights on from nine females. Only one out of nine wildtype (*wt*) female had stage IVa oocytes, while 4 out of 9 *mprs*$^{-/-}$ had stage IVa oocytes. (B) Further reduction in germinal vehicle breakdown (GVBD) in response to 5 nM exogenous 17α,20β-dihydroxy-progesterone (DHP) *in vitro* in stage IVa oocytes with all seven mPR paralogues (*mprs*$^{-/-}$) mutated. (C) Majority of stage IVa oocytes from *wt* underwent GVBD after a 3-hour incubation with 5 nM DHP *in vitro*. Oocyte become translucent following GVBD because of fusion of yolk protein that permits more lights going through. An immature oocyte failed undergoing GVBD is indicated by an arrow. (D) Many stage IVa oocytes from *mprs*$^{-/-}$ did not undergo GVBD following treatment with DHP for 3 hours. Immature oocytes failed undergoing GVBD are indicated by arrows. Scale bar: 1mm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Ovulation delay in *mprs*$^{-/-}$ females

In addition to impaired oocyte maturation, we also found impaired ovulation in *mprs*$^{-/-}$ females *in vivo*. Significant numbers of matured, but not yet ovulated follicles (stage IVb follicles) were only found in the ovaries of *mprs*$^{-/-}$ *in vivo* 30 minutes after lights were turned on but not in *wt* females (Figure 5.3A). Since nuclear progestin receptor (Pgr) is the key mediator for ovulation,

we determined Pgr expression in *mprs*$^{-/-}$ oocytes. The transcript levels of *pgr* were lower in stage III oocytes (21:00) and stage IVb oocytes (8:00) in *mprs*$^{-/-}$ compared to those in *wt* (Figure 5.3B). Meanwhile, the protein level of Pgr was also significantly reduced in stage IVa follicles in the *mprs*$^{-/-}$ (Figure 5.3C). Therefore, we checked the upstream (lhcgr (luteinizing hormone receptor)) and downstream (proteinases) gene expressions of Pgr and found reduced expression of *lhcgr*, *mmp2* (matrix metallopeptidase 2) in pre-ovulatory follicles in *mprs*$^{-/-}$ (Figure 5.4).

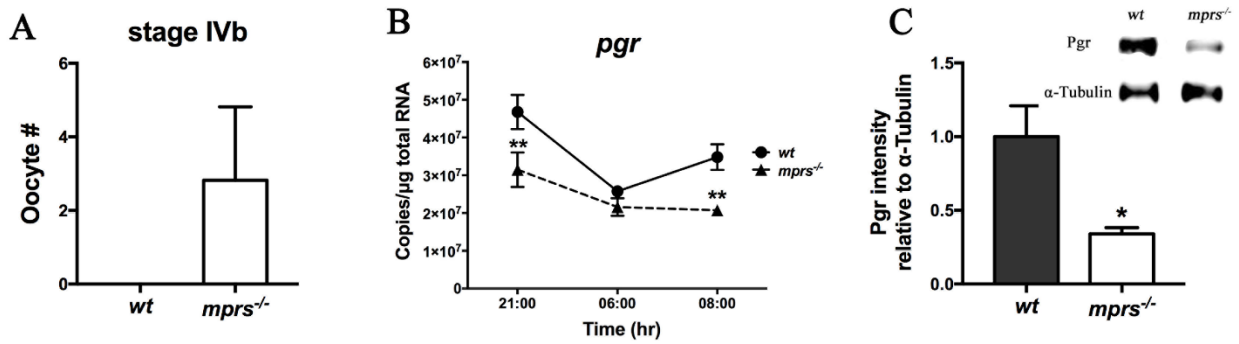


Figure 5. 3 Reduced ovulation due to attenuated expression of nuclear progestin receptor (Pgr) in all seven membrane progestin receptor paralogues mutant (*mprs*$^{-/-}$) *in vivo*. (A) Stage IVb oocytes were found in the ovaries of *mprs*$^{-/-}$ 30 minutes after lights on but not in wildtype (*wt*) females *in vivo*. N=9. (B) Reduced expression of *pgr* transcripts in most advanced oocytes in *mprs*$^{-/-}$ (stage III oocytes collected at 21:00, stage IVa oocytes collected at 6:00, stage IVb oocytes collected at 8:00. n=6). (C) Reduced expression of Pgr protein in the stage IVa oocytes from *mprs*$^{-/-}$ collected at 6:00 compared to those from *wt* (n = 3). **p* < 0.05; ***p* < 0.01.

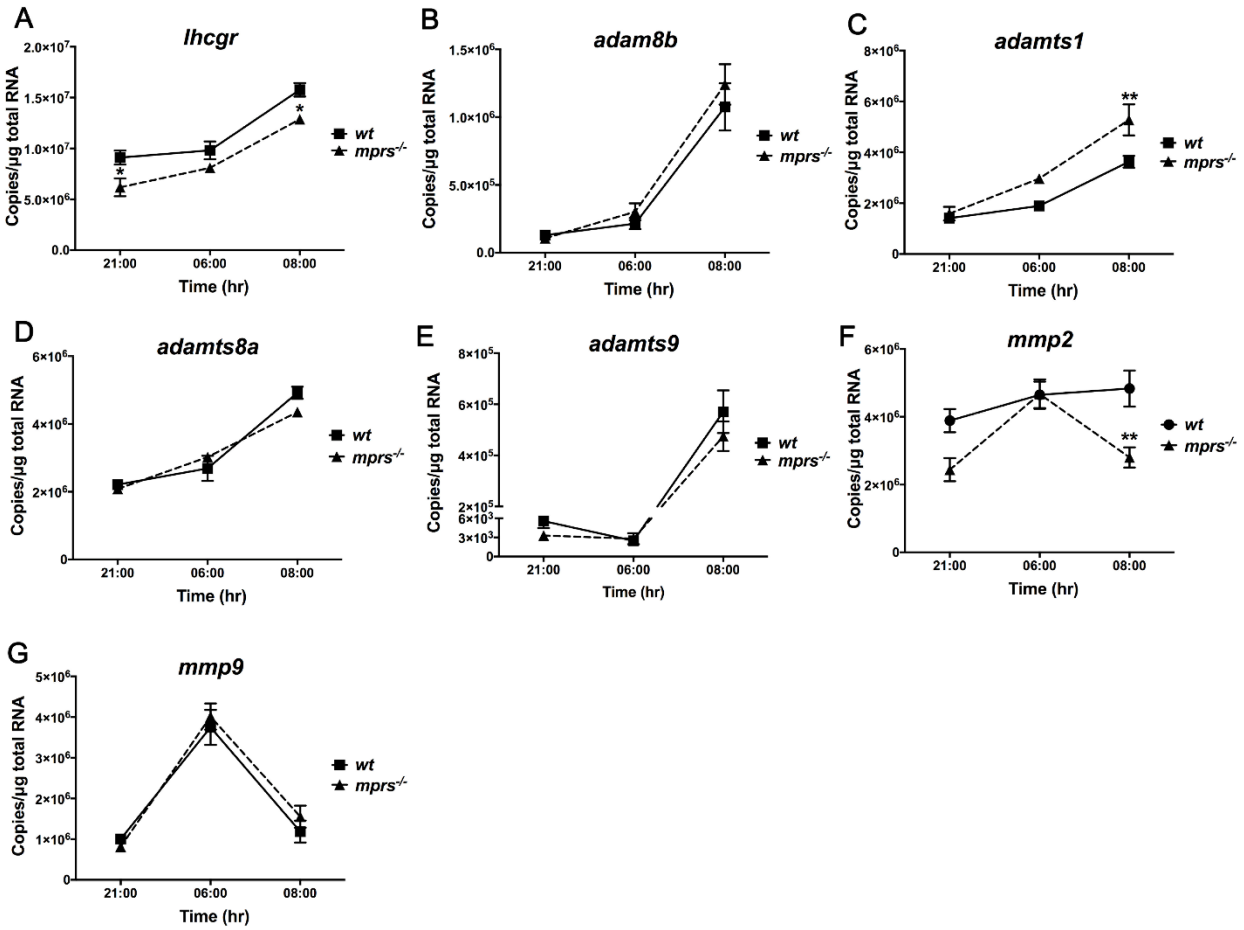


Figure 5. 4 Reduced expression of *luteinizing hormone* receptor (*lhcgr*) and metallopeptidases in most advanced oocytes in all seven membrane progestin receptor paralogues mutant (*mprs*$^{-/-}$; 21:00, stage III; 06:00, stage IVa; 08:00, stage IVb). Low *lhcgr* and *mmp2* expression can be observed in Stage IVb oocytes of *mprs*$^{-/-}$. In addition, expression of *adamts1* was upregulated in Stage IVb oocytes in *mprs*$^{-/-}$. (A) *lhcgr*; (B) *adam8b*; (C) *adamts1*; (D) *adamts8a*; (E) *adamts9*; (F) *mmp2*; (G) *mmp9*. Asterisks indicate a significant difference of transcripts compared to wildtype (*wt*) at the same time point. adam8b, a distintegrin and metalloproteinase domain 8b; adamts1, a disintegrin and metalloproteinase with thrombospondin type 1 motif 1; adamts8a; adamts9; mmp2, matrix metalloproteinase 2; and mmp9. *$p < 0.05$; **$p < 0.01$.

Reduced fertility in *mprs*$^{-/-}$ females

To evaluate the fertility of *mprs*$^{-/-}$, we conducted a continuous mating study using mature female fish (n=10) of 4-month-old with known fertile wildtype males over 2 weeks. The fecundity of mutant female zebrafish was remarkably compromised (*mprs*$^{-/-}$, 914.6 ± 105.8 fertilized embryos/2 weeks, *p=0.0039*) in comparison with *wt* females (1361 ± 83.88 fertilized embryos/2

weeks, n=10) (Figure 5.5A). In addition, we found that *mprs*$^{-/-}$ females (59.96 ± 7.69%) had low spawning frequency than those in *wt* females (71.43 ± 5.63%), though was not significant (Figure 5.5B). Cross anatomical examination revealed an obvious decrease in the size of ovaries in *mprs*$^{-/-}$ when compared with their *wt* littermates. Gonadosomatic index (GSI) was also smaller in *mprs*$^{-/-}$ than *wt* (Figure 5.5C). We observed mutant females always produced less embryos than *wt* females (Figure 5.5D). In addition, less stage V oocytes were obtained from *mprs*$^{-/-}$ than from *wt* (Figure 5.5E). All these evidences suggested fertility is compromised in *mprs*$^{-/-}$ females.

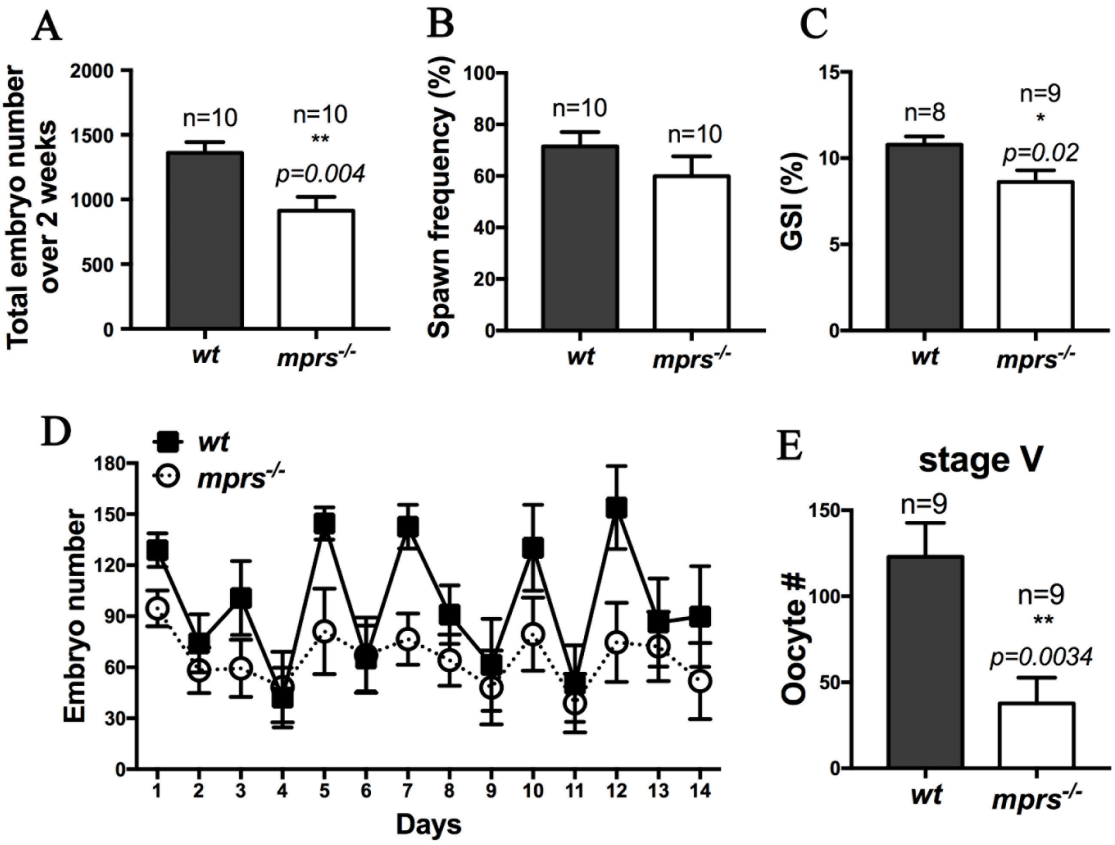


Figure 5. 5 Reduced fertility in all seven membrane progestin receptor paralogues mutant (*mprs*$^{-/-}$) females. (A) Fewer embryos were spawned in *mprs*$^{-/-}$ females when compared to wildtype (*wt*) during a two-week examination. (B) Spawning frequency in *mprs*$^{-/-}$ female was compromised, but not significantly. (C) Gonadosomatic index (GSI) of *mprs*$^{-/-}$ female was smaller than *wt*. (D) Daily fertilized embryos released from *mprs*$^{-/-}$ in comparison to *wt* (n=10). (E) Fewer Stage V oocytes were found in *mprs*$^{-/-}$ ovaries. *$p < 0.05$; **$p < 0.01$.

Poor-quality of zygotes from *mprs*$^{-/-}$ females

Not only the number, but also the quality of embryos is affected in *mprs*$^{-/-}$. Compared to embryos from *wt* incross, embryos from *mprs*$^{-/-}$ females cross with *wt* males had smaller yolks (Figure 5.6A). In addition, the embryos from *mprs*$^{-/-}$ female were restricted in a smaller chorion with narrow space for embryonic development (Figure 5.6B). The ratio of width of chorion to that of yolk was also significantly reduced in embryos from *mprs*$^{-/-}$ females when compared to those from *wt* females (Figure 5.6C). The yolk of some mutant embryos appeared dark and opaque under a dissecting microscope, and the cells divided in a disorganized pattern at a very early stage (8-16 cell stage) (Figure 5.6D). We found the yolk granules on the surface of early-stage embryos from *mprs*$^{-/-}$ females displayed a disorganized and scrambled pattern with multiple holes (Figure 5.6Ec & 5.6Ed) when compared to those from *wt* (Figure 5.6Ea & 5.6Eb). Embryos from *mprs*$^{-/-}$ males crossed with *wt* females showed normal appearance and development as those from *wt* (data not shown).

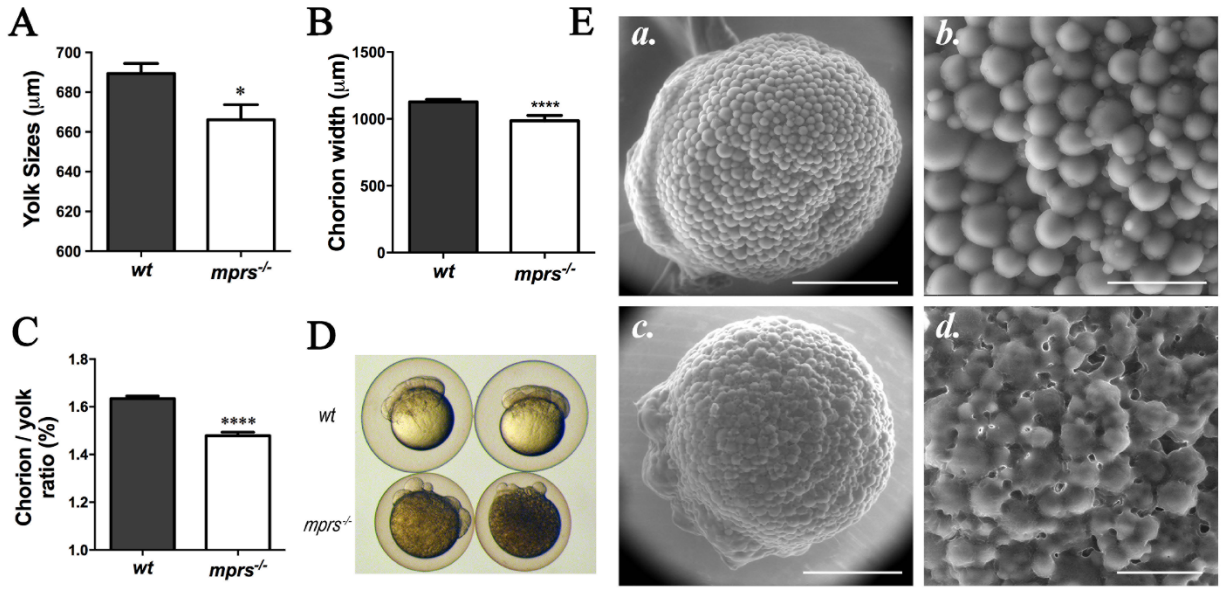


Figure 5. 6 Malformed embryos from in all seven membrane progestin receptor paralogues mutant (*mprs*$^{-/-}$) females. (A-C) Reduced yolk size, chorion width, and the ratio of chorion size to yolk size in the *mprs*$^{-/-}$ embryos. 30% epiboly embryos were used (n=7). (D) Representative

malformed embryos (16-cell stage) from *mprs*$^{-/-}$ females with smaller yolk and chorion. (E) Disorganized and scrambled yolk granules on the surface of *mprs*$^{-/-}$ early stage embryo (32-cell stage embryo). a & b) *wt*; c & d) *mprs*$^{-/-}$. Scale bars: a & c) 200 µm; b & d) 50 µm.

Abnormal early oogenesis in *mprs*$^{-/-}$ females

In addition to impaired oocyte maturation and ovulation, we found mPRs also had roles in early oogenesis. Higher percentage of stage I oocyte was observed in *mprs*$^{-/-}$ when compared to *wt* (Figure 5.7A). Compared to *wt* (Figure 5.7B), more stage I oocytes were observed in the ovaries of *mprs*$^{-/-}$ (Figure 5.7C).

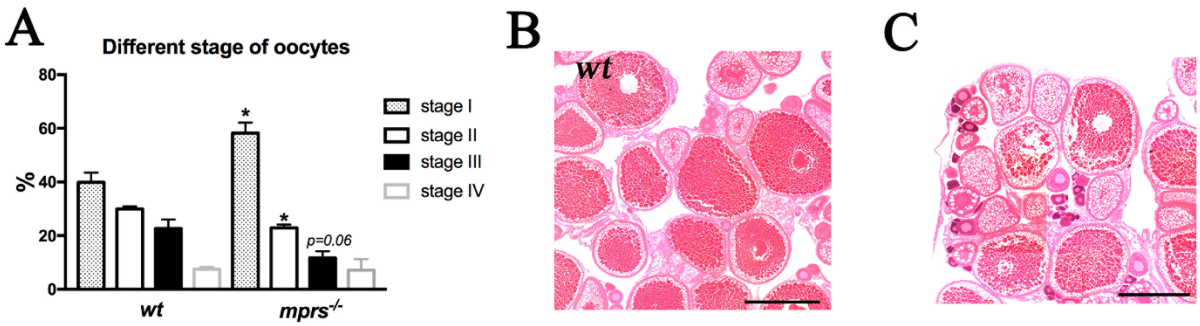


Figure 5. 7 Abnormal oogenesis in all seven membrane progestin receptor paralogues mutant (*mprs*$^{-/-}$) ovaries. (A). Higher percentage of stage I oocytes was found in *mprs*$^{-/-}$. (B) & (C). Representative image of wildtype (*wt*) or *mprs*$^{-/-}$ ovary. Scale bar: 1mm. *$p < 0.05$.

Discussion

Accumulating evidence suggest mPRα is the nongenomic progestin receptors involved in various physiological processes including oocyte maturation in several teleosts (Hanna et al., 2006; Hanna and Zhu, 2011; Shi et al., 2016; Tubbs et al., 2010; Zhu et al., 2003b). However, studies to date were focused mostly on mPRα. The functions of other mPR paralogues still remain unclear. Most critically, all the studies conducted to date were either using overexpression or knockdown approaches, evidence from knockouts are still lacking. To our knowledge, this is the first attempt to identify mPRs' functions in reproduction *in vivo* using gene editing method to knockout each

and all seven mPRs in one organism. Our study clearly demonstrated feasibility and advantages of alteration of multiple genes in a relative economic model, zebrafish. Our results clearly show that mPRs have functions in progestin induced oocyte maturation, ovulation, egg quality, and female fertility. Involvement of mPR signaling in meiosis resumption has been shown in other teleost species including goldfish (Tokumoto et al., 2006; Tokumoto et al., 2012), medaka (Roy et al., 2017), Atlantic croaker (Tubbs et al., 2010), European eel (Morini et al., 2017), and flounder (Shi et al., 2016). The involvement of mPRs in the regulation of ovulation, egg quality and female fertility were only first demonstrated in current study. Manipulation of mPRs signaling should affect female reproduction including oocyte maturation, ovulation and egg quality, which may have important implications for aquaculture. Progestin signaling is also important for various reproductive processes including endometrial preparation, follicular development, steroidogenesis, ovulation, implantation of fertilized egg, maintenance of pregnancy, breast development, and milk production in mammals (Dressing et al., 2010; King and Brucker, 2010; Macias and Hinck, 2012; Nilsson et al., 2006; Peluso, 2006; Peluso et al., 2008a). Further investigation on the roles and signaling of mPRs will advance our understanding and regulation of these important processes in mammals.

The mPRα and mPRβ are the membrane progestin receptors that mediate progestin signaling and induce oocyte maturation. Firstly, mPRα and mPRβ display a high affinitive (Kd~5 nM), saturable, displaceable, single-binding site specific for DHP (Hanna et al., 2006; Thomas et al., 2014; Valadez-Cosmes et al., 2016). Secondly, mPRα and mPRβ proteins have been localized on the plasma membranes of granulosa cell, theca cells, and oocytes (Dressing et al., 2010; Hanna and Zhu, 2011; Thomas, 2003). In addition, prior to oocyte maturation, in response to a dramatic increase in progestins, mPRα and mPRβ are up-regulated in fully-grown immature oocytes (Hanna

and Zhu, 2011; Thomas, 2003; Zhu et al., 2003b). Also, mPRs appear to function as plasma membrane-bound GPCRs mediating rapid actions of P4 *via* activation of an inhibitory G protein (Gi) and suppression of adenylyl cyclase activity and cAMP production (Hanna et al., 2006; Thomas et al., 2007). In a previous study, antisense microinjection of mPRα can block progestin induced oocyte maturation (Zhu et al., 2003b). Agreeing with this previous study, we also found impaired oocyte maturation *in vivo* and *in vitro* in this study. Overall, mPRs located on the oocyte membrane, bind progestin with high affinity, and are involved in the nongenomic action of progestin in oocyte maturation.

The functions and signaling of different membrane progestin receptors in oocyte maturation may not be identical. For example, signaling of zebrafish mPRα but not mPRβ was blocked by pertussis toxin, implying mPRβ activates a different pertussis toxin-insensitive G protein (Hanna et al., 2006). In addition, over-expression of mPRα in follicle-enclosed oocytes significantly increased the activity of MAPK, the production of cyclin B protein, and the number of oocytes that underwent oocyte maturation without exogeneous progestin, while over-expression of mPRβ alone had no such effect (Hanna and Zhu, 2011). We also investigated the different mPRs in oocyte maturation. Our results suggest that mPRα1 (Paqr7a), mPRα2 (Paqr7b), mPRβ (Paqr8), and mPRγ2 (Paqr5b), but not mPRγ1 (Paqr5a), mPRδ (Paqr6), and mPRε (Paqr9) play a role in oocyte maturation. In addition, the largest reductions in oocyte maturation were observed in oocytes from *mprα2*$^{-/-}$ and *mprγ2*$^{-/-}$. The mPRα2 was recognized as mPRα in zebrafish and other teleost species previously. Therefore, our results is consistent with those reported previously (Hanna et al., 2006; Hanna and Zhu, 2011; Shi et al., 2016; Zhu et al., 2003b). In contrast, the studies of mPRγ2 in oocyte maturation *in vitro* are still missing which need further investigation.

Due to genetic compensation in zebrafish (El-Brolosy et al., 2019; Ma et al., 2019), even mPRγ1 (Paqr5a), mPRδ (Paqr6), and mPRε (Paqr9) seem dispensable for maturation, these mPRs may still play a role in oocyte maturation. Therefore, we generated a mutant line with lacking all seven mPRs (*mprs*$^{-/-}$). However, even in mPRs total mutant (*mprs*$^{-/-}$), oocyte maturation still can occur slowly and incompletely. Other progestin receptors besides mPRs may explain why oocyte maturation still can happen in *mprs*$^{-/-}$. Besides mPRs, there are two progestin receptor families, Pgr and Pgrmcs. Around 5% of the Pgr can be associated with the plasma-membrane caveolae (Norman et al., 2004). Pgr's palmitoylation, a post-translational modification that may be important for membrane anchoring of Pgr (Pedram et al., 2007). Acceleration of oocyte maturation by overexpression of Pgr in *Xenopus* and zebrafish oocytes also suggest the involvement of Pgr in meiosis resumption (Bayaa et al., 2000; Hanna et al., 2010; Tian et al., 2000). Pgr may participate in oocyte maturation through interacting with SRC tyrosine kinase and subsequent activation of the MAPK pathway (Zhang et al., 2008). However, we found acceleration of oocyte maturation in three different Pgr knockout lines (unpublished data). Therefore, the role of Pgr in oocyte maturation remain controversial. The Pgrmcs may also play important roles in oocyte maturation. Pgrmc1 antibody injection significantly lowered oocyte maturation percentages after 24 hours of culture (Luciano et al., 2010). Similar results were found in *pgrmc1*$^{-/-}$ female zebrafish (Wu et al., 2018). But Pgrmc1 may not regulate oocyte maturation directly, as no evidence is available on the direct interaction between Pgrmc1 and G-protein. Pgrmc1 might facilitate expression and plasma localization of mPRα, which in turn regulates oocyte maturation (Thomas et al., 2014; Thomas et al., 2007; Wu et al., 2018).

Interestingly, around 20% of oocytes from *wt* females spontaneously matured without the ligand, DHP, while none of the oocytes from *mprs*$^{-/-}$ matured under same condition. In yeast, mPRs

can constitutively transduce signals without ligand when expressed at high levels, but did require an agonist when expressed at low levels (Kupchak et al., 2009; Smith et al., 2008; Villa et al., 2009). Right before oocyte maturation, mPRs are up-regulated and/or activated and then the net production of the second messenger increases above the threshold necessary to initiate downstream signaling molecules that initiate oocyte meiosis in *wt* zebrafish (Hanna and Zhu, 2011). In contrast, spontaneous oocyte maturation could not occur due to lack of mPRs and their signals in *mprs*$^{-/-}$. Therefore, mPRs may be important for spontaneous oocyte maturation.

No evidence has shown mPRs located in nucleuses and functioned as transcription factors. However, nongenomic actions through mPRs may eventually affect the genomic action of progestin. Mutation of mPRs may affect nongenomic action through MAPK signaling pathway (Pace and Thomas, 2005), which can eventually affect gene transcription and translation, resulting in lower level transcription and translation of nuclear progestin receptor (Pgr). Studies have shown Pgr is essential for ovulation across different species (Kubota et al., 2016; Lydon et al., 1995; Zhu et al., 2015). Then, low expression levels of Pgr in mPRs total mutants cause the ovulation delay *in vivo*. Similar low Pgr level and impaired ovulation was found in *pgrmc1/2*$^{-/-}$ (Wu and Zhu, 2019). The previous study also showed progestin membrane receptor-mediated pathways can regulate transactivation of Pgr resulting in an alteration in gene transcription (Karteris et al., 2006).

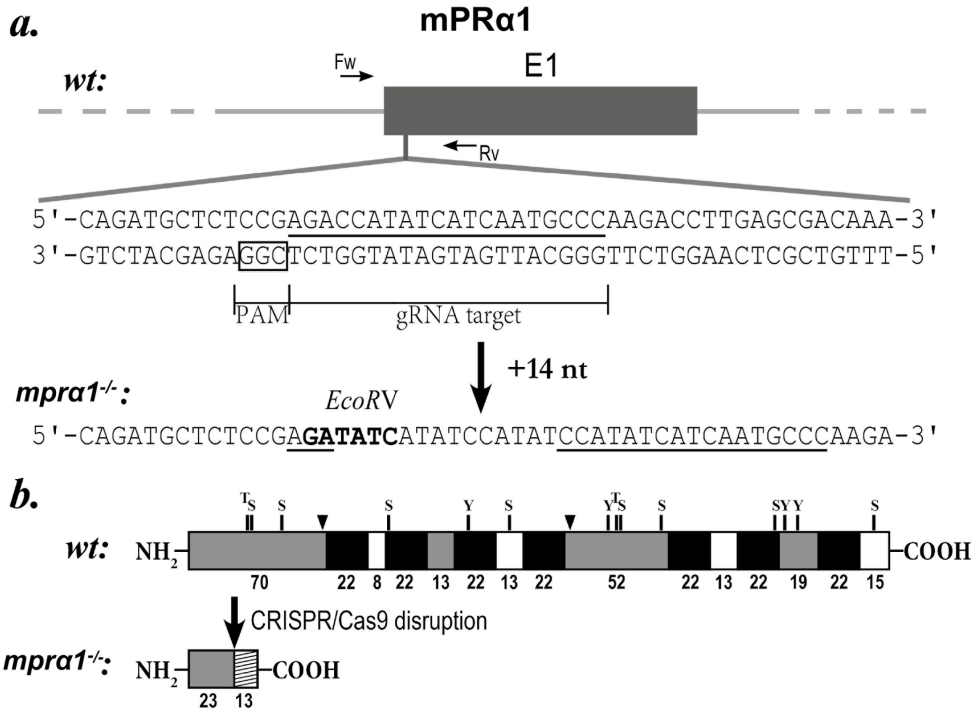
Ovulation normally began around 8:00 (one hour before lights on). In order for ovulation to occur, nuclear progestin receptor (Pgr) and its related signaling molecules need to be expressed at appropriate levels around 8:00. Although the Pgr transcript was not significantly different in *mprs*$^{-/-}$ compared to its level in *wt* at 6:00 (Figure 5.3B), the Pgr protein was clearly lower in *mprs*$^{-/-}$ knockout than its expression in *wt* at 6:00 (Figure 5.3C). The low transcript levels of genes including *pgr* and *lhcgr* at 21:00 (the night before spawning) and 8:00 (the morning prior to the

spawning) may indicate overall low basal expression of genes in *mprs*$^{-/-}$. This low expression of Pgr may further reduced protein expression of Pgr and its downstream molecules important for ovulation. It would be ideal to determine the protein levels and enzyme activities of these genes, which will be the topics of future studies when research reagents and tools such as antibodies and enzymatic assays become available.

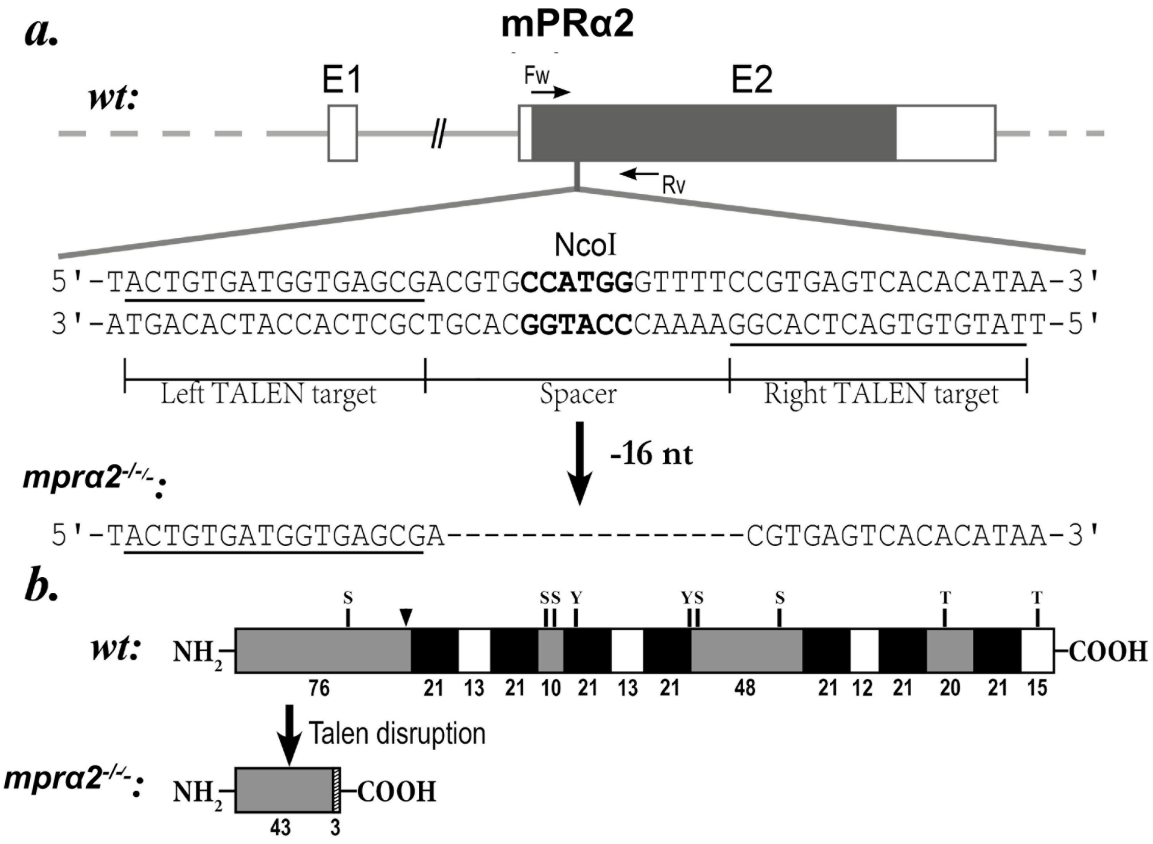
Besides oocyte maturation and ovulation, we also found significant more stage I oocytes in *mprs*$^{-/-}$ females (Figure 5.7). This indicates impaired development in early oogenesis in *mprs*$^{-/-}$ oocytes. Similar phenotype was also found in *pgrmc1*$^{-/-}$ and *pgrmc2*$^{-/-}$ mutant zebrafish (Wu et al., 2019; Wu et al., 2018). During early stages of oogenesis in Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*), DHP significantly promotes DNA synthesis in the ovarian germ cells and acted directly on the initiation of the first meiosis of oogenesis (Miura et al., 2007). Pgrmcs and mPRs may play an important role in promoting early oogenesis; however, further studies are needed.

Overall, our results showed mPRs are important for oocyte maturation and ovulation. However, further studies are required to identify the additional membrane progestin receptors for oocyte maturation in zebrafish since oocyte maturation could still occur even all seven paralogs of mPR were mutated.

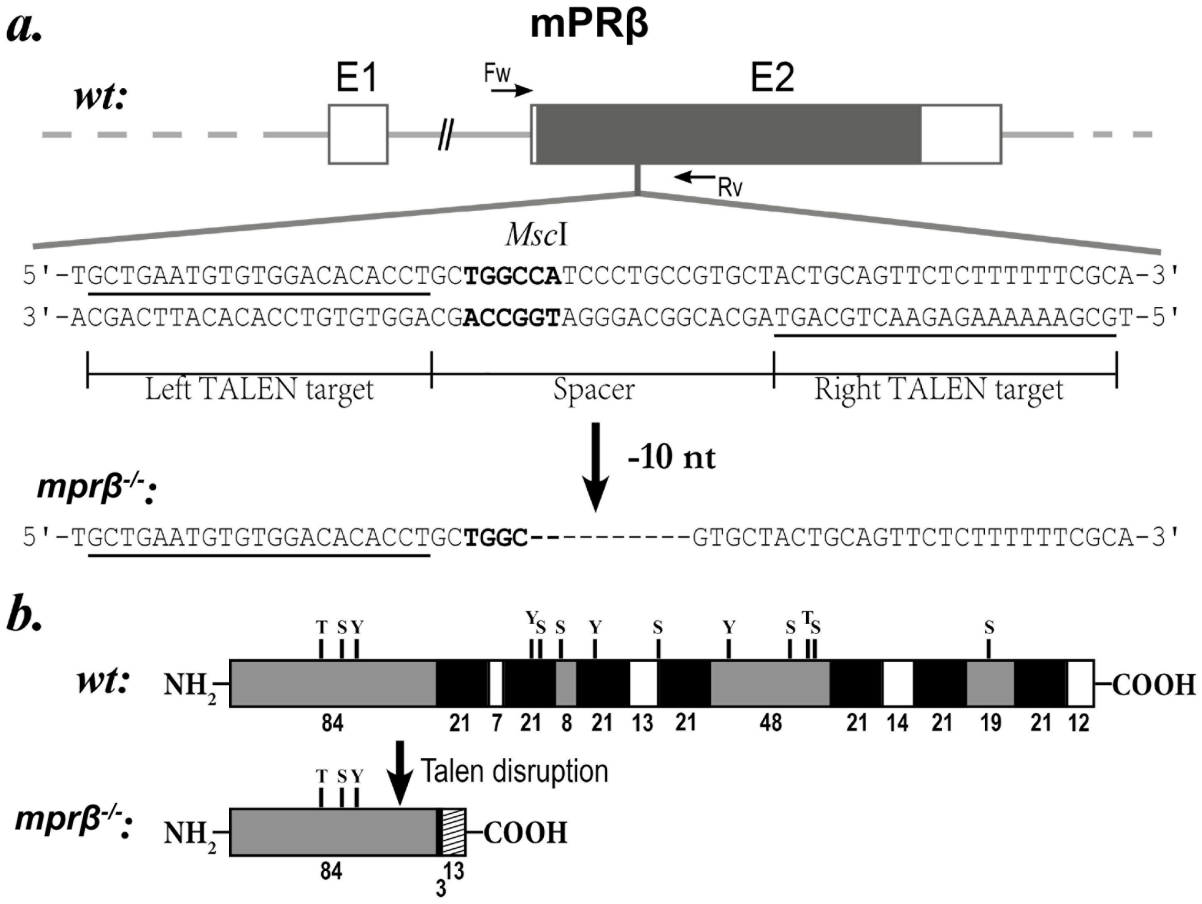
Supplemental Figure 5. 1 Generation of mPRα1/Paqr7a (Accession number: A0A0R4I9I9) global mutant lines using CRISPR/Cas9. a) Genomic structures of the zebrafish mPRα1 gene, CRISPR/Cas9 **targets, and representative mutation. Exons are shown as boxes in proportion to their sizes and introns simply as a solid line with double slashes. Grey box represents a coding region. The binding site of gRNA is underlined. For mPRα1, an EcoRV restriction enzyme site was unexpectedly generated after the disruption.** Fw, Forward PCR primer; Rv, Reverse PCR primer. b) Predicated truncated mPRα1 proteins from *mpra1*$^{-/-}$, lacking seven transmembrane domains. Extracellular (gray), seven-transmembrane (solid black), and cytoplasmic (clear) domains were predicted by TMpred program. Vertical lines indicate potential phosphorylation sites. ▼, potential N-linked glycosylation site. Numbers above: amino acids from the N-terminal end; numbers below: number of amino acids in each domain. Domains with slashes represent disrupted amino acid sequences in *mpra1*$^{-/-}$**.**



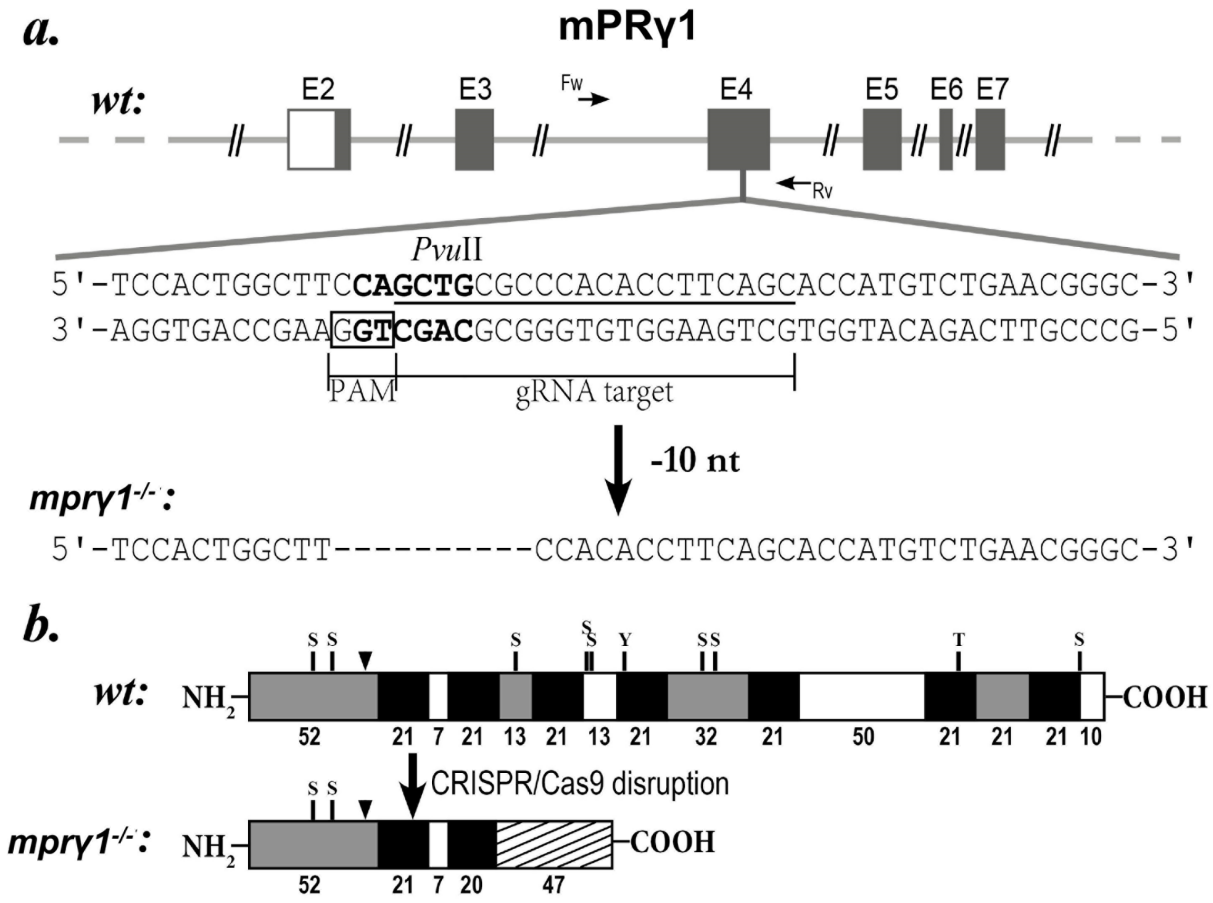
Supplemental Figure 5. 2 Generation of mPRα2/Paqr7b (Accession number: Q801G2) global mutant lines using TALENs. a) Genomic structures of the zebrafish mPRα2 gene, TALEN targets, and representative mutation. Exons are shown as boxes in proportion to their sizes and introns simply as a solid line with double slashes. Grey box represents a coding region. The binding sites of TALENs are underlined. A restriction enzyme cleavage site highlighted in bold is incorporated around each target. Fw, Forward primer; Rv, Reverse primer. b) Predicated truncated mPRα2 proteins, lacking seven transmembrane domains. Extracellular (gray), seven-transmembrane (solid black), and cytoplasmic (clear) domains were predicted by TMpred program. Vertical lines indicate potential phosphorylation sites. ▼, potential N-linked glycosylation site. Numbers above: amino acids from the N-terminal end; numbers below: number of amino acids in each domain. Domains with slashes represent disrupted amino acid sequences in *mprα2*$^{-/-}$.



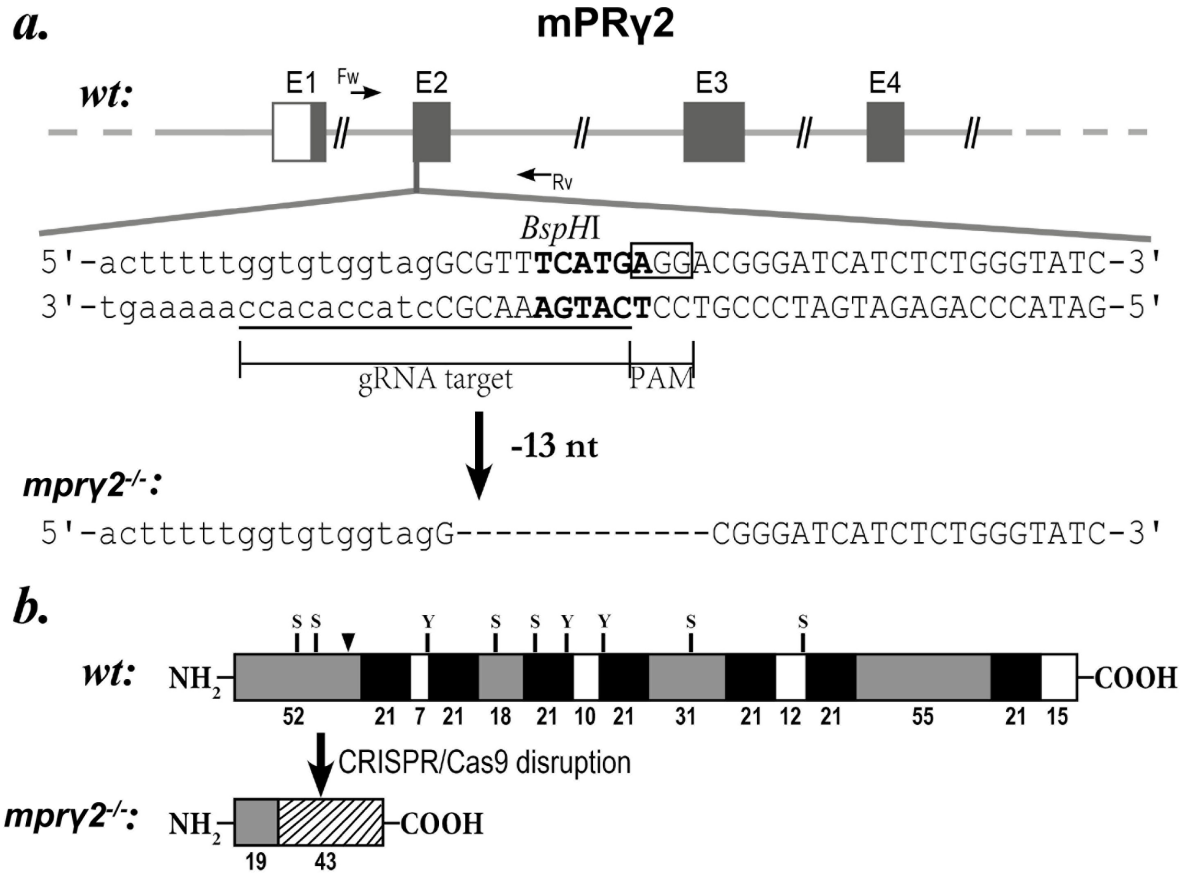
Supplemental Figure 5. 3 Generation of mPRβ/Paqr8 (Accession number: Q8TEZ7) global mutant lines using TALENs. Similar details can be found in supplemental figure 5.2 legend.



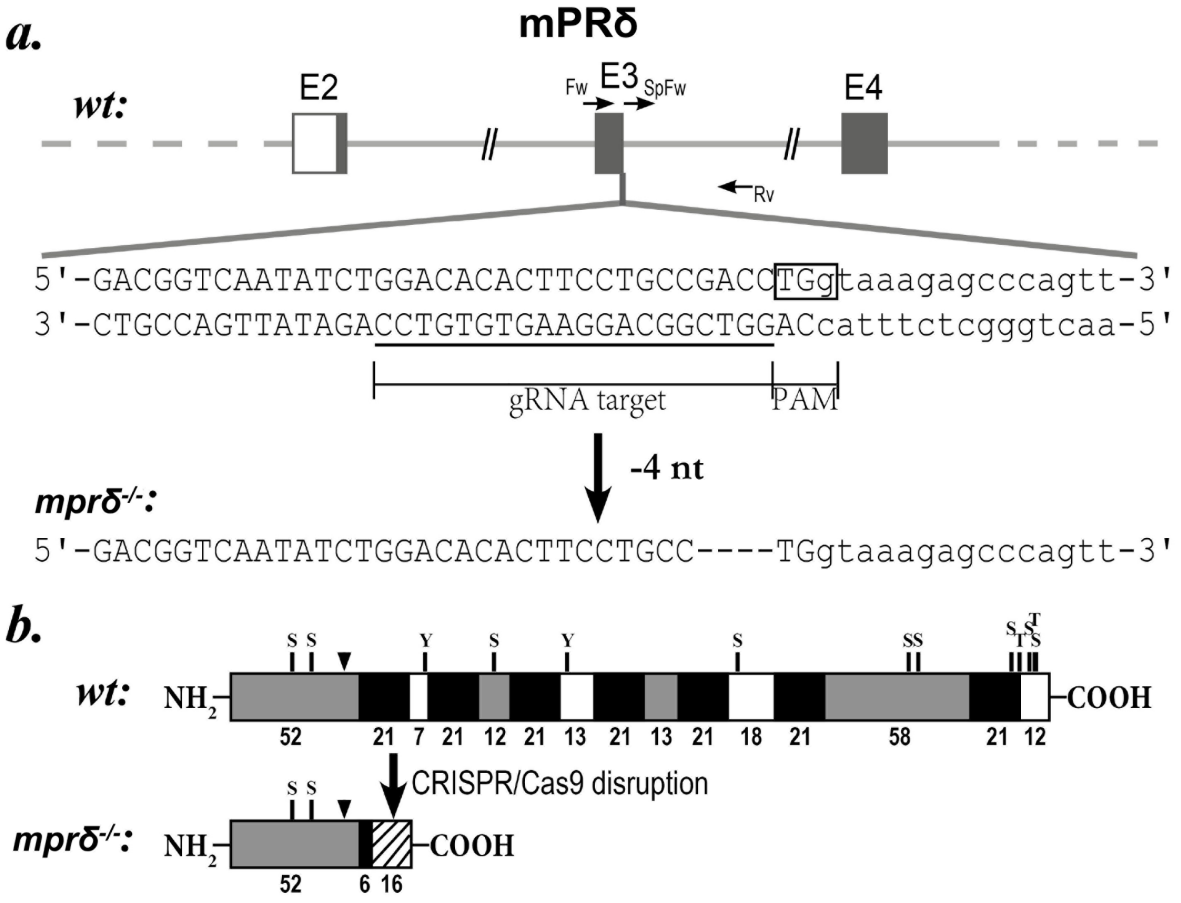
Supplemental Figure 5. 4 Generation of mutant lines of mPRγ1/Paqr5a (Accession number: Q7ZVH1) using CRISPR/Cas9. Similar details can be found in supplemental figure 5.1 legend.



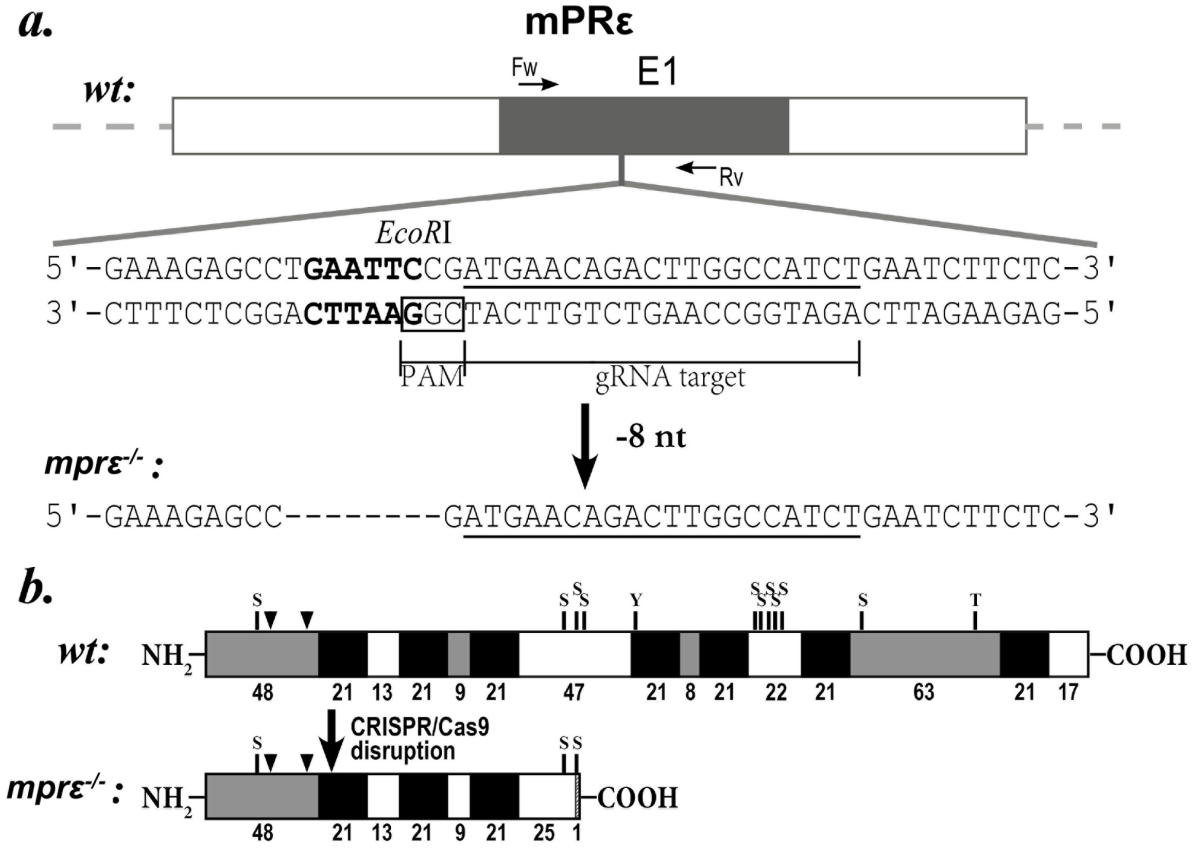
Supplemental Figure 5. 5 Generation of mPRγ2/Paqr5b (Accession number: Q6DC77) global mutant lines using CRISPR/Cas9. See supplemental figure 5.1 legend for other details.



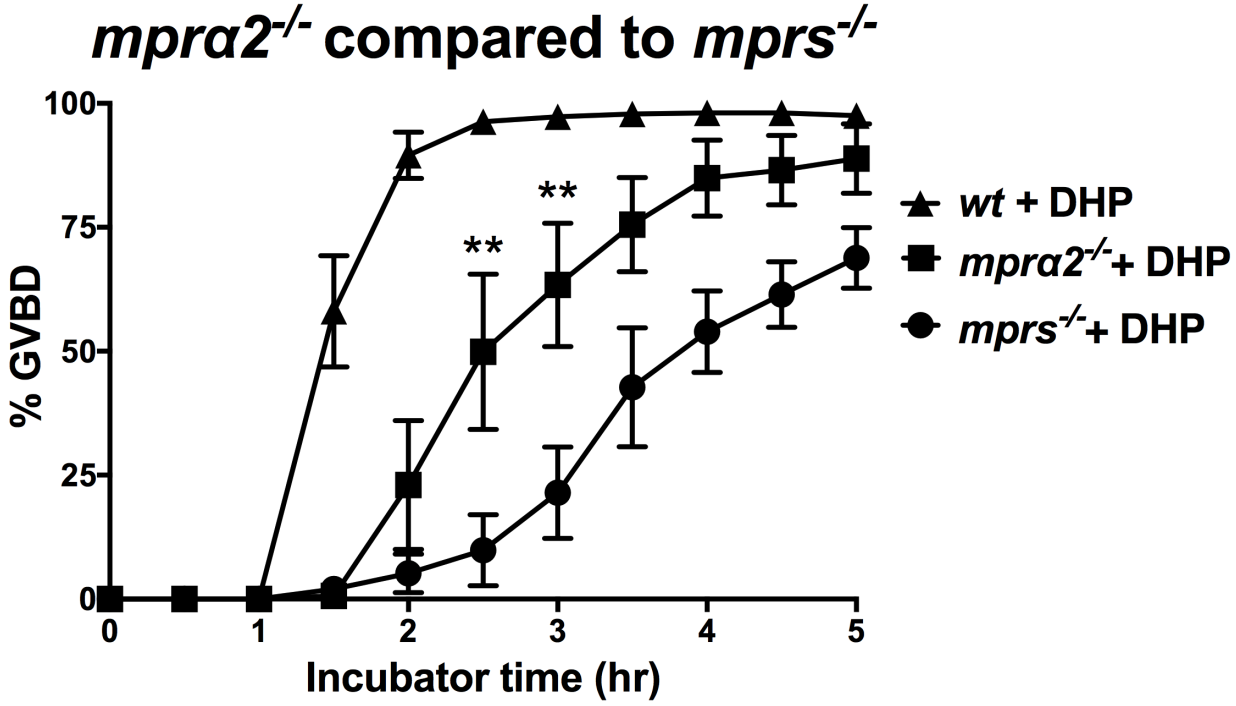
Supplemental Figure 5. 6 Generation of mPRδ/Paqr6 (Accession number: Q6TCH4) global mutant lines using CRISPR/Cas9. For detection mPRδ mutant, specific primer sets for *wild-type* or *mutant* was used (Supplemental table 5.1). Fw, Forward PCR primer; Rv, Reverse PCR primer; SpFw (E), Specific forward primer. Please refer supplemental figure 5.1 legend for other details.



Supplemental Figure 5. 7 Generation of mPRε/Paqr9 (Accession number: Q6ZVX9) global mutant line using CRISPR/Cas9. For more detail information, see supplemental figure 5.1 legend.



Supplemental Figure 5. 8 Further reduction of progestin induced germinal vesicle breakdown (GVBD) in stage IVa fully-grown immature oocytes in *mprs*$^{-/-}$ compared to those in *mpr*α*2*$^{-/-}$. Stage IVa immature oocytes were exposed to 5 nM exogenous 17α,20β-dihydroxy-progesterone (DHP) and incubated at room temperature for up to 5 hours, rate of GVBD were determined every 30 minutes. * denote significant difference in the GVBD rate compared between *mpr*α*2*$^{-/-}$ and *mprs*$^{-/-}$ (*$p < 0.05$; **$p < 0.01$).



Supplemental Table 5. 1 Sequences of PCR primers used for genotyping.

Gene	Forward primer	Reverse primer	Wildtype-specific reverse primer	Mutant-specific reverse primer
mPRα1	CCAGATCCACAAATCTTAAC	AGAAGTGGCCAGGAATGAG		
mPRα2	GTTGTGATGGAGCAGATTGG	CGGCCAGGAGTAAGATGAAC		
mPRβ	CTTTGTCAGGCAGAGGTATG	CACACCAACGTAGTCTACAA		
mPRγ1	CCACTGGGTGTTTACTCATTTGT	AAGACAGAACCCCAAGCAGG		
mPRγ2	ACAGAAGTTAGAGCAAGCAA	AGGTGCCCTTTAACTACATC		
mPRδ	CTGTTTCAGTGTTTGCATGT	ATGAACTGCCAGATAGAACC	TGGACACACTTCCTGCCGA	GACACACTTCCTGCCTGGT
mPRε	TCCCCAACTACAGCCTACGG	GGAGAAACGCGTTCTGGGAT		

Supplemental Table 5. 2 Percentages of oocyte maturation treated with DHP over 5 hours.
Mean ± SEM.

Time(hr)	*wt*	*mPRα1*$^{-/-}$	*mPRα2*$^{-/-}$	*mPRβ*$^{-/-}$	*mPRγ1*$^{-/-}$	*mPRγ2*$^{-/-}$	*mPRδ*$^{-/-}$	*mPRε*$^{-/-}$
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	1.33±1.33	0.00	0.00	0.00	0.00	0.00	0.71±0.71
1.00	0.00	20.57±9.00	0.00	0.00	1.79±1.79	0.00	1.48±1.48	0.71±0.71
1.50	58.07±11.23	62.15±9.50	0.62±0.62	49.04±16.25	57.46±4.01	52.05±15.00	46.80±6.12	59.60±14.8(
2.00	89.51±4.69	77.18±7.50	23.03±13.00	68.83±10.93	83.27±4.85	64.45±14.27	89.88±4.47	82.54±9.88
2.50	96.31±2.13	79.50±6.96	49.88±15.67	78.55±9.40	91.59±2.81	69.56±13.96	92.86±5.42	89.78±6.26
3.00	97.33±1.34	80.14±5.65	63.39±12.42	79.93±9.10	93.23±2.68	73.75±10.35	92.86±5.42	90.91±6.08
3.50	97.84±1.13	86.47±4.44	75.53±9.49	81.04±9.32	95.13±2.74	77.47±9.03	95.96±2.59	92.24±4.95
4.00	98.10±1.11	85.49±4.74	84.90±7.69	84.06±8.79	98.55±1.45	78.91±8.70	96.06±2.51	94.19±3.83
4.50	98.10±1.11	88.20±5.77	86.51±7.01	83.03±9.06	98.55±1.45	81.78±6.83	96.86±1.92	96.14±2.78
5.00	98.10±1.11	88.20±5.77	88.89±6.99	84.23±9.05	98.55±1.45	83.37±7.33	97.63±1.59	96.14±2.78

Supplemental Table 5. 3 Percentages of oocyte maturation treated with vehicle over 5 hours.
Mean ± SEM.

Time(hr)	*wt*	*mPRα1*$^{-/-}$	*mPRα2*$^{-/-}$	*mPRβ*$^{-/-}$	*mPRγ1*$^{-/-}$	*mPRγ2*$^{-/-}$	*mPRδ*$^{-/-}$	*mPRε*$^{-/-}$
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
0.50	0.00	0.00	0.00	0.00	0.91±0.91	0.00	0.00	0
1.00	0.00	0.61±0.61	0.00	0.00	1.68±1.03	0.00	0.00	0
1.50	0.64±0.64	0.61±0.61	1.04±0.67	0.00	5.44±0.71	0.00	0.00	3.61±0.20
2.00	0.64±0.64	0.61±0.61	1.04±0.67	1.33±1.33	8.21±1.12	0.00	1.72±1.72	3.58±0.23
2.50	2.92±1.86	5.00±3.11	3.13±1.16	2.00±2.00	11.63±1.29	0.91±0.91	1.72±1.73	6.00±0.58
3.00	3.66±2.47	5.00±3.11	6.54±2.79	2.00±2.01	12.79±0.95	3.29±1.42	7.76±7.76	9.02±0.56
3.50	4.99±3.67	11.31±5.37	9.16±3.13	4.39±2.22	13.78±1.56	9.16±4.04	10.32±8.65	11.00±0.58
4.00	5.84±4.01	15.69±7.54	12.70±3.80	7.24±3.09	16.80±2.95	9.16±4.05	10.39±8.64	11.00±0.58
4.50	6.49±4.23	18.77±7.45	15.27±4.90	10.33±5.16	17.30±2.80	10.12±4.55	13.25±10.31	11.67±0.88
5.00	6.73±4.43	21.88±8.45	17.48±5.74	11.19±5.80	21.31±4.57	10.12±4.56	13.25±10.31	11.67±0.88

Supplemental Table 5. 4 Percentages of oocyte maturation treated with DHP or vehicle over 5 hours. Mean ± SEM.

Time(hr)	*wt*+DHP	*mprs*$^{-/-}$+DHP	*wt*+Vehicle	*mprs*$^{-/-}$+vehicle
0.00	0	0	0	0
0.50	0	0	0	0
1.00	0	0	1.18±1.18	0
1.50	21.40±7.08	2.00±2.00	1.18±1.18	0
2.00	69.62±7.97	5.18±3.88	1.18±1.18	0
2.50	90.09±4.75	9.87±7.17	1.18±1.18	0
3.00	97.27±2.72	21.47±9.23	1.18±1.18	0
3.50	97.27±2.72	42.74±12.02	2.35±1.44	0
4.00	97.27±2.72	53.92±8.22	9.88±6.23	0
4.50	97.27±2.72	61.44±6.62	15.11±9.63	0
5.00	97.27±2.72	68.84±6.14	24.20±16.82	0

CHAPTER 6: Characterization of Oocyte Maturation and Ovulation in Zebrafish Lacking Pgrmcs and mPRs

Chapter summary

It is well-known progestin and membrane progestin receptors signaling are essential for meiosis resumption and oocyte maturation. Intriguingly, some of these stage IVa fully-grown, immature oocytes from single or combined knockouts of Pgrmcs or mPRs still could undergo final oocyte maturation even though oocyte maturation were greatly delayed and reduced. It is entirely possible that nongenomic progestin signaling required for oocyte maturation is mediated by multiple familes of progestin receptors including Pgrmcs and mPRs. Knocking out all members of both Pgrmc and mPR families may block meiosis resumption and final oocyte maturation. Therefore, a mutant line lacking all members of the Pgrmc and mPR families was generated by crossing *pgrmc1/2*$^{-/-}$ with *mprs*$^{-/-}$. Surprisingly, no adult female could be found in this total knockout (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$, N=127). In comparison, the sex ratios in other single or combined mutant lines (*pgrmc1*$^{-/-}$, *pgrmc2*$^{-/-}$, *pgrmc1/2*$^{-/-}$, *mprs*$^{-/-}$, and *pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) are similar to those found in wildtype fish. Since early estradiol exposure can increase the number of females in adult zebrafish, we continuously treated *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ zebrafish with estradiol (5 nM) from 20 dpf for 1 month and rescued some *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ females. This indicates Pgrmcs and mPRs are essential for survival of primary oocytes during early stage sex determination but not in adult female gonads. Interestingly, these females still can spawn when crossed with *wt* males, which indicate oocyte maturation and ovulation can still occur without Pgrmcs and mPRs. In addition, progestin may not induce oocyte maturation through Pgr since acceleration of oocyte maturation found in Pgr knockout lines. Therefore, progestins may bind with other unknown progestin receptor(s) to induce oocyte maturation.

Introduction

Progestins play essential roles in oocyte maturation in fish and frogs (Kalinowski et al., 2004; Pace and Thomas, 2005). So far, all three families of progestin receptors (Pgr, Pgrmcs, and mPRs) have been shown to be involved in oocyte maturation. Around 5% of Pgr is located on the membrane because of palmitoylation modifications of Pgr (Pedram et al., 2007). Also, human PGR contains a polyproline motif in the amino-terminal domain (aa 421-428) that interacts with the SH3 domain of Src and mediates rapid activation of c-Src and downstream MAPK (Boonyaratanakornkit et al., 2008). Injection of human PGR-B RNA into *Xenopus* oocytes resulted in the acceleration of the rate of progesterone-induced maturation (Boonyaratanakornkit et al., 2001). Similarly, microinjection of Pgr transcripts into immature oocytes accelerated oocyte maturation in zebrafish oocytes (Hanna and Zhu, 2011). Importantly, this acceleration of maturation in the Pgr injected group requires the genomic signaling pathway since maturation acceleration was blocked by treatment with the transcription inhibitor actinomycin D (Hanna and Zhu, 2011).

Pgrmc1 likely facilitates oocyte maturation by regulating the oocyte plasma membrane localization of mPRα, the receptor mainly responsible for mediating oocyte maturation and meiosis resumption in fish (Aizen et al., 2018). On the other hand, Pgrmc1 was also been suggested to be involved in estrogen maintenance of zebrafish oocyte meiotic arrest, *via* regulating G protein-coupled estrogen receptor 1 (GPER) -dependent epidermal growth factor receptor (EGFR) signaling (Aizen and Thomas, 2015). A reduced number of oocytes undergoing meiotic maturation both *in vivo* and *in vitro* was found in our Pgrmc1 zebrafish knockout line (*pgrmc1*$^{-/-}$). This reduced sensitivity to progestin corresponds well with significant reduced expression of mPRα (Wu et al., 2018). Studies of Pgrmc2 are still rare. Reduced oocyte maturation was found

in our Pgrmc2 zebrafish mutant line (*pgrmc2*$^{-/-}$) *in vivo* (Wu et al., 2019). Nonetheless, oocytes from *pgrmc2*$^{-/-}$ had similar sensitivity to DHP compared with wildtype *in vitro*. Pgrmc2 may be involved in DHP synthesis because DHP levels in *pgrmc2*$^{-/-}$ ovaries showed a significantly decrease compared to those in *wt*. Thus, Pgrmc2 regulates oocyte maturation *via* regulating DHP synthesis (Wu et al., 2019).

The third family of the progestin receptors, mPRs appear to function as membrane-bound GPCRs mediating rapid actions of progestin *via* activation of an inhibitory G protein (Gi) and suppression of adenylyl cyclase activity and cAMP production during oocyte maturation. Progesterone, DHP, or 20βS significantly activated MAPK in mPRα and mPRβ transfected cells within 5 minutes of stimulation (Hanna et al., 2006). Furthermore, cAMP levels of both receptors transfected cell lines dropped significantly in response to DHP in a dose and time-dependent manner (Hanna et al., 2006). Over-expression of mPRα also increases cyclin B production in follicle-enclosed oocytes concomitantly with the increase in maturation (Hanna and Zhu, 2011). Antisense microinjection of mPRα and (or) mPRβ can block progestin-induced oocyte maturation (Zhu et al., 2003b). For 7 paralogs of mPRs in zebrafish, we found impaired oocyte maturation (GVBD ratio) in zebrafish lacking mPRα1 (*paqr7a*$^{-/-}$), mPRα2 (*paqr7b*$^{-/-}$), mPRβ (*paqr8*$^{-/-}$), or mPRγ2 (*paqr5b*$^{-/-}$). In contrast, no difference was observed in oocyte maturation in the single knockout of mPRγ1 (*paqr5a*$^{-/-}$), mPRδ (*paqr6*$^{-/-}$), or mPRε (*paqr9*$^{-/-}$) (Wu and Zhu, unpublished data). In addition, mutating all seven mPRs (*mprs*$^{-/-}$) reduced oocyte maturation *in vivo* and *in vitro* in zebrafish (Wu and Zhu, unpublished data).

Since the impaired oocyte maturation in both *pgrmc1/2*$^{-/-}$ and *mprs*$^{-/-}$ stage IVa oocytes, nongenomic progestin signaling required for oocyte maturation may be mediated through multiple classes of progestin receptors including Pgrmcs and mPRs. Therefore, we generated combined

knockouts of Pgrmcs and mPRs (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) but could not find any females. After being treated with estradiol for one-month, partial female *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ can be rescued. But these female *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ are still fertile, indicating oocyte maturation and ovulation still can happen in *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ females.

Materials and methods

Zebrafish lines

The wildtype zebrafish (*Danio rerio*) strain used in this investigation, Tübingen strain, was initially obtained from the Zebrafish International Resource Center, then propagated in our lab at East Carolina University. Zebrafish lacking both Pgrmcs and mPRs were generated by crossing of *pgrmc1/2*$^{-/-}$ with *mprs*$^{-/-}$. Since *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ don't have females, this mutant line was maintained by crossing male *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ with female *pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$. Fish were kept under a photoperiod of 14h light and 10h dark (lights on at 09:00, lights off at 23:00), with water temperature around 28.5℃, pH ~7.2, and salinity conductivity ranging from 700 to 1,200 μS in automatically controlled zebrafish rearing systems (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed twice a day and supplied with newly hatched brine shrimp. All the animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

Rescue of mutant phenotype by E2 treatment

Mutant fish were exposed continuously to estradiol (E2) for 30 days from 20 to 50 dpf to determine whether E2 exposure would restore normal sex ratio in a combined knockout of Pgrmcs and mPRs. Briefly, the juvenile zebrafish at 20 dpf (*wt* or mutants) were cultivated in two 10-L tanks containing E2 or control carrier (ethanol). Both mutants (*pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ or

pgrmc1$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) zebrafish were from the same breeding pairs of males (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) and females (*pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) parents, hosted together in the same tank. A 10 μl stock E2 (5 mM in 100% ethanol) was added to the exposed tanks (final conc 5 nM). Half of the tank water was replaced daily, and fresh E2 was added to maintain the same exposure concentration. The hormone exposure was stopped at 30 days following the exposure. All the fish were transferred back into the zebrafish husbandry system, and were raised to mature adults. Sex was determined individually following dissection and morphological observation of gonads at three-month post-fertilization. The genotype of each fish was determined using genomic DNA extracted from tail fin and specific PCR amplification described previously (Wu et al., 2018).

GVBD assay

To examine the role of Pgr in oocyte maturation, ovarian follicles from three different Pgr knockout lines were isolated and incubated to determine the sensitivities of follicles to a maturation inducing steroid, DHP (Hanna and Zhu, 2011; Pang and Thomas, 2009). Gravid female zebrafish were euthanized and their ovaries were immediately dissected out. Then, the ovaries were washed several times in 60% Leibovitz L-15 medium (Sigma-Aldrich). The individual ovarian follicles were carefully separated without damaging the follicular cell layers based on previously established protocols (Hanna and Zhu, 2011). Fully-grown immature oocytes were randomly selected and distributed into the wells of a 24-well plate (~30 follicles/well). The final concentration for DHP and ethanol in the treatment group was 5 nM and 0.1%, respectively. As a control, 1 μl pure ethanol was added into control wells containing 1 ml medium and the same number of follicles (~30 follicles/well) collected at the same time from the same group of individual fish. The follicles were incubated in a vehicle or DHP for 5 hours, with GVBDs being

scored every half hour during the incubation period. To confirm the results, all the experiments were repeated five times.

Genotyping

Genomic DNA was extracted by incubating zebrafish tail fin sample in 50 mM NaOH (50 µl) at 95 °C for 15 min (Meeker et al., 2007). To differentiate *pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ zebrafish from *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ zebrafish, a mutant-specific reverse primer was then designed according to the mutated sequence in CRISPR/Cas9-induced mutation (*Pgrmc1*-Forward: 5'-GGGGGTGTCTGAAAGGAACT-3' *Pgrmc1*-wildtype Reverse: 5'-CTCCTCAACCGGGCCATAGT-3' *Pgrmc1*-mutant Reverse: 5'-GGCTCCTCAACCGAGACAAA-3'). PCR condition was optimized for efficient identification of the specific mutation. After an initial denaturation for 2 minutes at 94 ℃, the cycling reaction was performed with the profile of 30 s at 94 ℃, 30 s at 61 ℃ and 45 s at 72 ℃ for 35 cycles, followed by a 10-min extension at 72 ℃ with a Thermal Cycler (Eppendorf, Hauppauge, NY, USA).

Histological analysis and sex determination

The entire fish (35 or 45 dpf zebrafish) or dissected gonads (60 or 90 dpf zebrafish) were immediately fixed in 10% buffered formalin solution overnight followed by dehydration and infiltration. Samples were embedded and processed for paraffin sectioning using a microtome (Leica Reichert Jung Biocut 2030). Paraffin sections of 10 µm were mounted on slides, deparaffinized, rehydrated and washed with deionized water. Then the sections were stained with hematoxylin and eosin (H&E), dehydrated, mounted and viewed under the Olympus BX41 microscope (Olympus, Japan). All stained sections were captured with Olympus DP72 and viewed through cellSens (Olympus, Japan).

Statistical analysis

All the results were presented as mean ± SEM. Significant differences among paired groups were determined using unpaired Student's *t* test (GraphPad Prism 7.0a, San Diego, CA, USA). Statistical significance was set at $p<0.05$.

Results

All *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ fish developed into males

To our surprise, no female was found in zebrafish with *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ around 90 dpf. In contrast, wildtype and other mutant lines had both males and females with similar sex ratios (Figure 6.1A). Histological examination of the *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ testis exhibited normal spermatogenesis. These males are fertile when tested by crossing with *pgrmc1*$^{+/-}$*pgrmc2*$^{-/-}$ *mprs*$^{-/-}$ females.

During early stage gonad development, 71.43% of total knockout (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$, N=21) zebrafish had male biased gonads with early spermatocytes at 35 dpf, while only 30.77% wildtype contained early spermatocytes at this time point (Figure 6.1B). At 45 dpf, 86.21% of total knockout (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) fish (N=29) were male (Figure 6.1C), and this ratio increased to 100% by 60 dpf (N=92) (Figure 6.1D). In comparison, the male ratios of wildtype zebrafish were 41.38% at 45 dpf and 53.38% at 60 dpf, respectively. In conclusion, our novel results suggest that a redundant function exists between the Pgrmc family and mPRs family in oocyte survival in zebrafish.

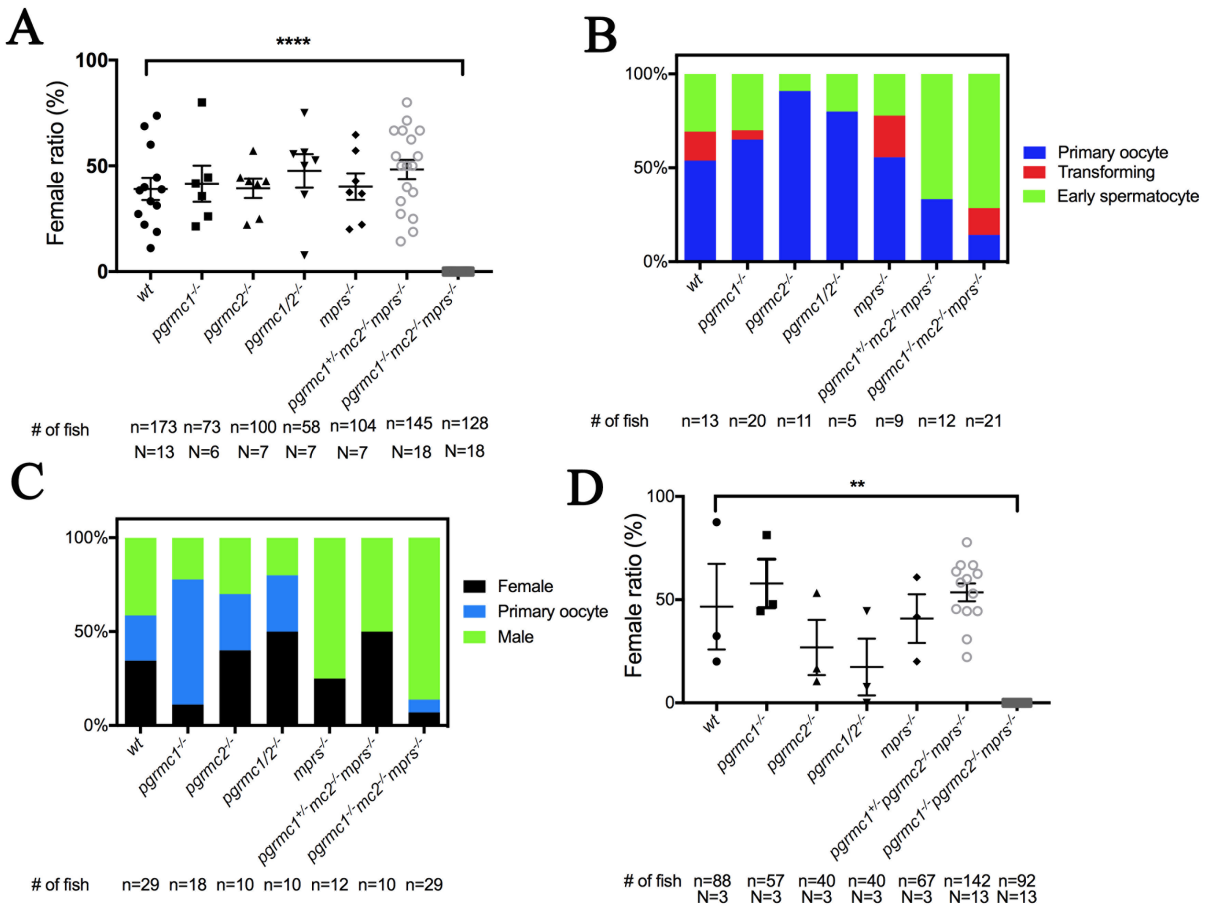


Figure 6. 1 Sex ratio during gonadal development in individual or combined knockouts of all members of Pgrmc and mPR families. (A) Sex ratio at 90 dpf. (B) Sex ratio at 35 dpf. (C) Female ratio at 45 dpf. (D) No adult female was found at 60 dpf when all members of Pgrmcs and mPRs were mutated (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$). Significant difference in sex ratio was found between *wt* and combined knockouts of Pgrmcs and mPRs mutants (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$). **, p<0.01; ***, p<0.001.

Rescue of mutant females by estradiol (E2) treatment

To further investigate the roles of Pgrmcs and mPRs in oocyte maturation and ovulation, sexually mature females are needed. Therefore, we rescued the male-only phenotype of *pgrmc1*$^{-/-}$ *pgrmc2*$^{-/-}$*mprs*$^{-/-}$ by E2 treatment (5 nM) during the period of gonadal differentiation (20 dpf-50 dpf). Compared to the ethanol treatment group, exposure to E2 led to normal ovarian formation in

some *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ females (Figure 6.2). These females are fertile as tested by crossing with wildtype control males.

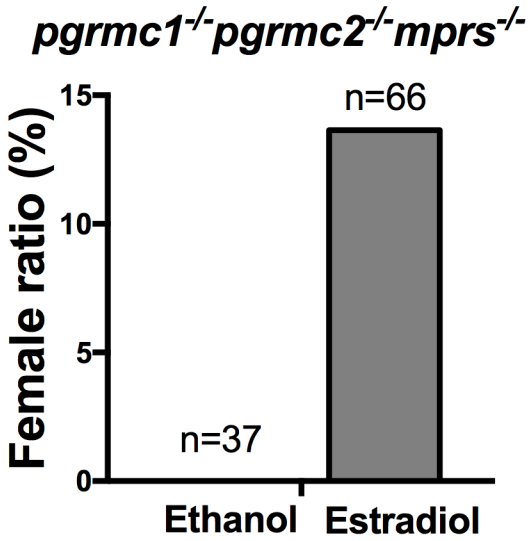


Figure 6.2 Estradiol treatment rescued around 15% of *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ adult females while ethanol group had no female.

Oocyte maturation acceleration in *pgr*$^{-/-}$ stage IVa oocytes *in vitro*

Since the female fish lacking all members of Pgrmcs and mPRs families still can undergo oocyte maturation, we hypothesized progestins may induce oocyte maturation through the last known progestin receptor-Pgr. Contrary to our prediction, all three Pgr-deficient lines (*pgr2d*$^{-/-}$, *pgr15*$^{-/-}$, and *pgr35*$^{-/-}$) shown accelerated oocyte maturation when compared to *wt* in GVBD assays (Figure 6.3). No difference can be found in groups treated with ethanol (Figure 6.3).

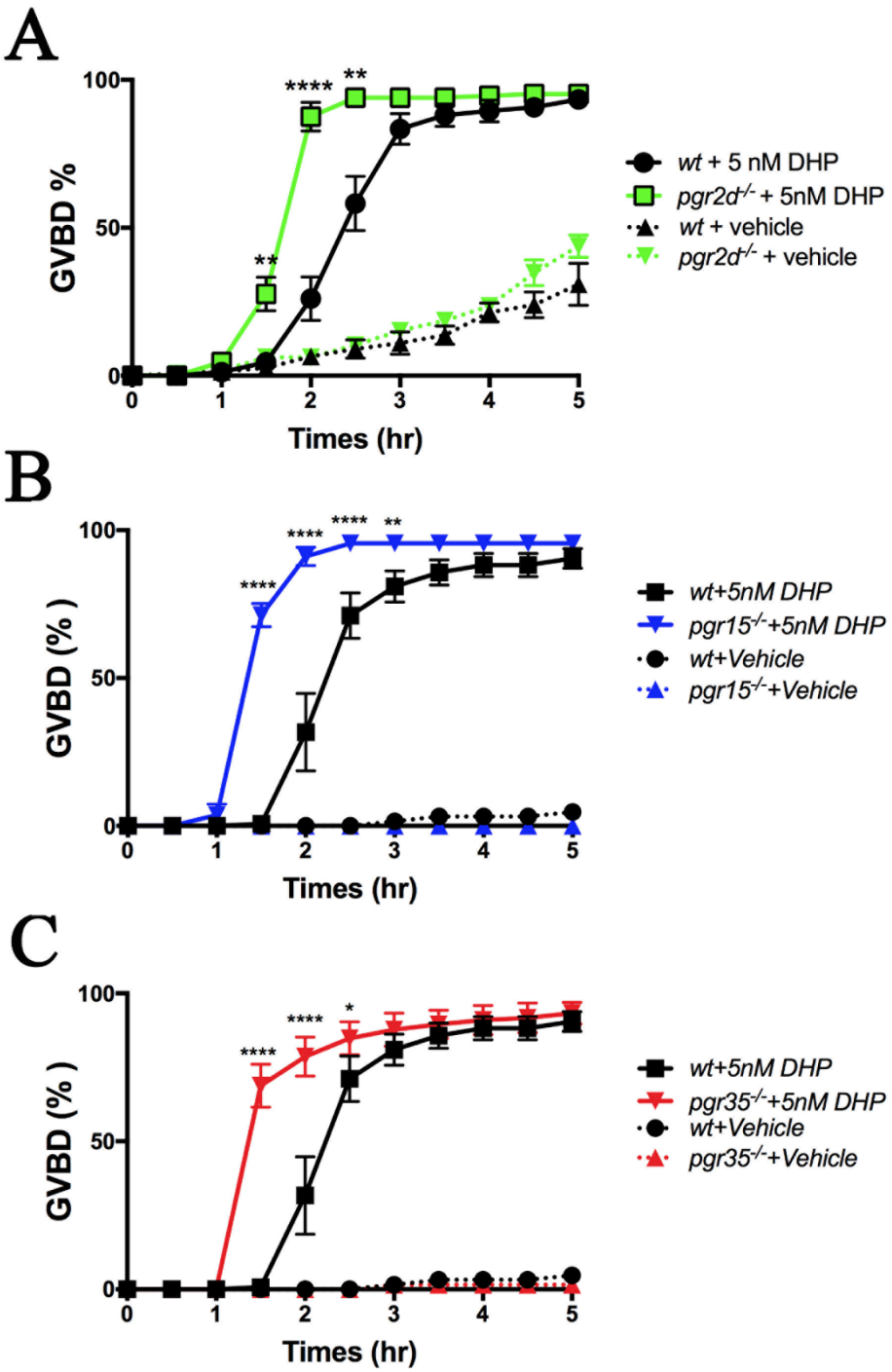


Figure 6. 3 Acceleration of GVBD in three different Pgr knockout zebrafish lines. GVBD assays *in vitro* in three different Pgr knockout lines. Stage IVa oocytes were treated with 5nM DHP or vehicle for 5 hrs. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.

Discussion

Pgrmcs and mPRs in sex determination

Gonadal differentiation in zebrafish is highly plastic and can be affected by internal (genetic background and hormones) and external factors (temperature, hypoxia, and density) (Abozaid et al., 2012; Hill Jr and Janz, 2003; Liew et al., 2012; Liew and Orbán, 2013; Orban et al., 2009; Shang et al., 2006). Zebrafish first develop ovary-like gonads or juvenile ovaries, which are characterized by the initiation of meiosis around 10 dpf followed by development of perinucleolar oocytes and ovarian cavity (Takahashi, 1977). The formation of testis therefore involves apoptosis of perinucleolar oocytes followed by transformation of the gonad into testis (Uchida et al., 2002). Estrogens are known to be important in promoting ovarian development and maintaining femaleness afterward (Guiguen et al., 2010). Zebrafish treated with aromatase inhibitor during gonadal differentiation or knocking out aromatase resulted in 100% masculinization (Fenske and Segner, 2004; Lau et al., 2016). Another female hormone, progesterone, is well known for reproductive processes such as oocyte maturation and ovulation in vertebrates (Robker et al., 2000; Thomas, 2012). However, determining the physiological functions of these progesterone receptors *in vivo* is challenging due to gene duplication among progesterone receptor families and overlapping functions of these receptors. To overcome this obstacle, single as well as combined knockouts for Pgrmcs and mPRs were generated. Surprisingly, no female can be found in zebrafish lacking both Pgrmcs and mPRs. This is the first time in any species that progestin receptors play a role in sex determination. Pgrmcs and mPRs did not seem to affect the differentiation of the juvenile ovary into the mature ovary; however, they are essential for the formation of so-called juvenile ovary and survival of oocyte-like germ cells. Progesterone plays an anti-apoptotic role in ovarian cells (Peluso JJ, 2003, Peluso JJ, 2004, Peluso JJ et al., 2005,

Peluso JJ, 2007). Both Pgrmc and mPRs have all been suggested to play important roles in progesterone's anti-apoptotic functions (Dressing et al., 2010; Peluso, 2006; Peluso et al., 2010). Losing Pgrmcs and mPRs may cause a higher apoptosis level which leads to all male phenotype. The mechanism for Pgrmcs and mPRs in the sex determination of zebrafish needs further investigation.

Pgr's functions in oocyte maturation

Two nuclear progestin receptors (XPR-1 and XPR-2) have been identified in *Xenopus* (Bayaa et al., 2000; Tian et al., 2000). Overexpression of XPR-1 or XPR-2 accelerates progesterone induced oocyte maturation and cell cycle re-entry, while XPR-1 antisense inhibits the process (Bayaa et al., 2000; Tian et al., 2000). Overexpression of Pgr increases rapid intracellular nongenomic signaling in response to progestins resulting in activation of MAPK and cell cycle regulators such as cyclins in both *Xenopus* oocytes and human breast cancer cells (Bayaa et al., 2000; Boonyaratanakornkit et al., 2007; Faivre et al., 2005; Skildum et al., 2005; Tian et al., 2000). These *in vitro* experiments indicate Pgr may promote oocyte maturation. However, Pgr plays an inhibitory role in zebrafish oocyte maturation because oocyte maturation accelerated in our Pgr knockout lines. After incubation with DHP for 90 mins, an approximate 50% reduction in Pgr transcript was observed in follicle-enclosed oocytes (Hanna and Zhu, 2011). In addition, *pgr* transcript was absent in stage IV oocytes, diminishing Pgr's potential role in oocyte maturation (Hanna and Zhu, 2011). Overall, the role of Pgr in oocyte maturation remains controversial.

Oocyte maturation independent of Pgrmcs and mPRs

Since oocyte maturation still happens in *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$, and oocyte maturation sped up in Pgr knockouts, unknown progestin receptor(s) may present on oocytes that may also contribute(s) to oocyte maturation.

CHAPTER 7: Summary and Future Directions

In summary, I have demonstrated that both members of Pgrmc and mPR families play roles in several processes of female reproduction including nongenomic progestin signaling-induced oocyte maturation and genomic progestin-induced ovulation. I have provided the following evidence to support the involvement of Pgrmcs and mPRs in oocyte maturation in zebrafish: oocyte maturation delay and reduction *in vivo* in *pgrmc1*$^{-/-}$, *pgrmc2*$^{-/-}$, *pgrmc1/2*$^{-/-}$, and *mprs*$^{-/-}$; stage IVa oocytes from *pgrmc1*$^{-/-}$, *pgrmc1/2*$^{-/-}$, and *mprs*$^{-/-}$ have decreased sensitivities to the oocyte maturation hormone DHP. Pgrmc1 itself doesn't bind with the G-protein, but Pgrmc1 can upregulate mPRα2 protein expression and therefore increase G-protein signaling and promote oocyte maturation (Figure 7.1). Mutation of Pgrmc2 did not directly affect oocytes' maturation, but Pgrmc2 mutant could decrease progestin (DHP) level in ovaries resulted in reduced oocyte maturation *in vivo* (Figure 7.1). Not all mPRs are involved in oocyte maturation. Mutatans of mPRα1, mPRα2, mPRβ, or mPRγ2 had delayed and reduced oocyte maturation (GVBD ratio) in zebrafish. In contrast, no difference was observed in oocyte maturation in the single knockout of mPRγ1, mPRδ, or mPRε (Figure 7.1). Further experiments focusing on the downstream signal pathway (etc. cAMP, cyclin B, CDC25) will elucidate underlying mechanisms of Pgrmcs and mPRs in oocyte maturation .

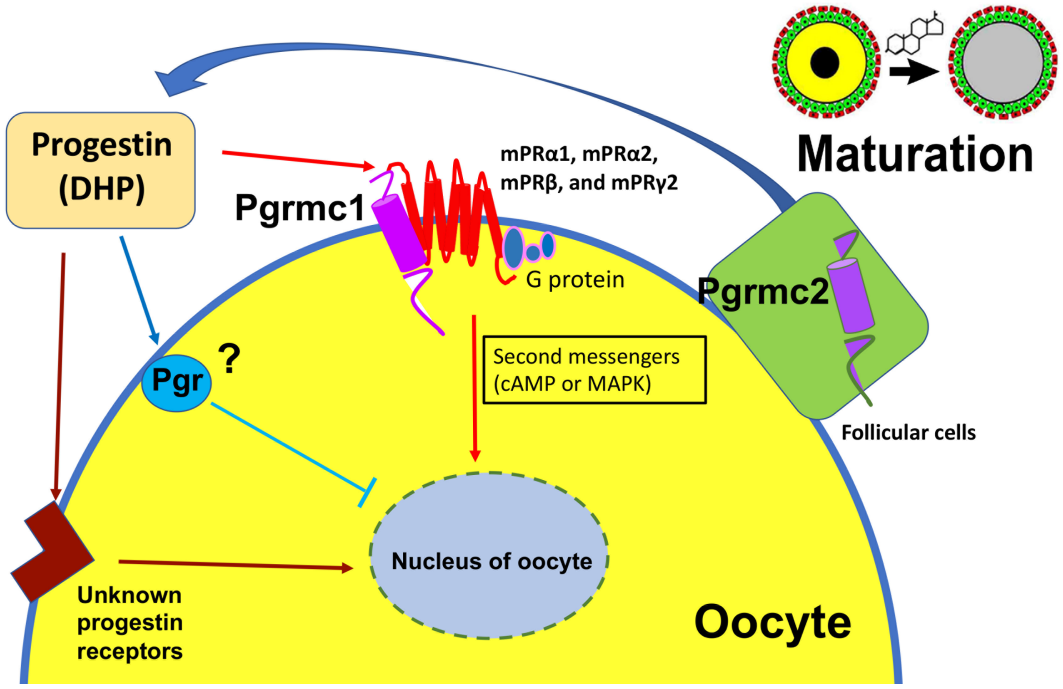


Figure 7. 1 Proposed progestin receptors responsible for oocyte maturation in zebrafish. mPRα1, mPRα2, mPRβ, mPRγ2 promote oocyte maturation in zebrafish. In contrast, role of mPRγ1, mPRδ, or mPRε in oocyte maturation in zebrafish is less clear. Pgrmc1 not only traffic mPRα to the oocyte membrane, but also can increase the expression of mPRα and therefore promotes oocyte maturation. Pgrmc2 increases oocyte maturation *via* increase progestin (DHP) synthesis . Pgr may play an inhibitory role in oocyte maturation.

In addition, Pgrmcs and mPRs also participate in genomic progestin signaling-induced ovulation: ovulation delay and reduction were found in *pgrmc1/2*$^{-/-}$; ovulation tardiness in *mprs*$^{-/-}$ *in vivo*; low Pgr level in *pgrmc1/2*$^{-/-}$ and *mprs*$^{-/-}$ oocytes resulted in low proteinase levels and caused reduced ovulation *in vivo* (Figure 7.2). While I have identified that Pgrmcs and mPRs can affect Pgr expression and therefore ovulation, the signaling pathways that Pgrmcs and mPRs affect Pgr expression are still not clear. Both Pgrmcs and mPRs are membrane proteins and lack DNA binding domains thus they may not bind to the promoter regions of Pgr directly and affect Pgr expression. Since Pgrmc1 can also be detected inside the nucleus, Pgrmcs may interact with other transcription factors and affect gene expressions (Peluso and Pru, 2014). It will be interesting to

identify the transcription factors that Pgrmcs can bind with. Also, Pgrmcs may affect a wide range of gene expression, which need to be further investigated for their roles in ovulation. The mPRs are located on the cell membrane, but not in the nucleus. Therefore, they may affect Pgr expression through secondary messages cAMP. The cAMP may affect gene expression through cAMP response element binding protein (CREB). It is necessary to investigate if CREB interact with the promotor of Pgr. Additional studies focusing on the interactions between different families of progestin receptors are required.

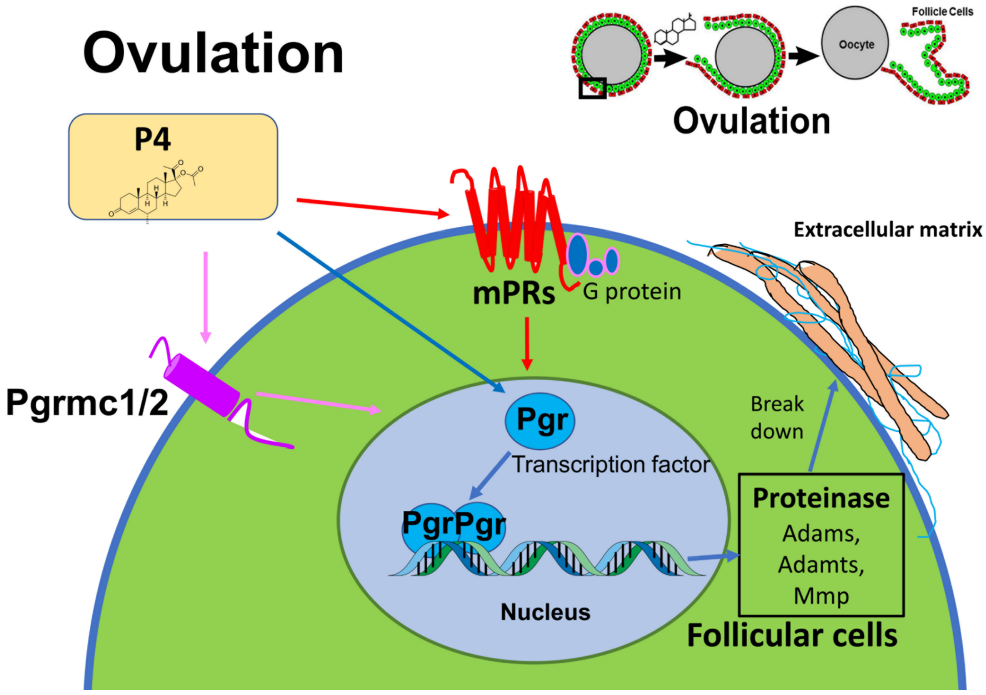


Figure 7. 2 Proposed progestin receptors responsible for ovulation in zebrafish. Pgr is essential for ovulation *via* regulation of proteinase expressions. Pgrmcs and mPRs also promote ovulation *via* regulation of Pgr expression.

In this study, I showed that Pgrmc2 increases steroid synthesis, especially DHP synthesis. Pgrmc1 may also has a role in steroid synthesis alike Pgrmc2, as the two paralogous share high sequence homology and heme binding domain. A previous study showed Pgrmc1 bounds

cytochromes P450 and affects cholesterol synthesis in the HCT116 cancer cell line (Kabe et al., 2016). However, evidence of Pgrmc involvement in steroid synthesis is still scarce. Further studies are required to understand the effects and mechanisms of Pgrmcs in steroid syntheses. For example, measuring other hormone levels besides DHP; using immunoprecipitation and proteomics, we can identify if Pgrmc binds with hormone synthesis enzymes; checking hormone synthesis enzyme activities when silence or overexpress Pgrmc.

A zebrafish mutant line lacking both families of Pgrmc and mPR (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$) was generated to circumvent overlapping functions between Pgrmc and mPR families. We hypothesized oocyte maturation and ovulation will be further impaired in *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ females. To our surprise, no female adults could be found in this 9-progestin-receptor-mutant zebrafish, indicating an important role of Pgrmcs and mPRs in sex determination in zebrafish. In a rescuing experiment with exogenous estradiol exposure, some female *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ zebrafish can be rescued and are fertile. This result suggests oocyte maturation still can occur without Pgrmcs and mPRs. In addition, nuclear progestin receptor (Pgr) had an inhibitory role in oocyte maturation since oocyte maturation was accelerated in Pgr knockout lines compared to *wt*. One possible scenario is presence of additional uncharacterized progestin receptor(s), which may also be responsible for progestin induced oocyte maturation. An alternative most likely scenario is oocyte maturation occurred *via* non-progestin signaling pathways *in vivo* in the 9-progestin-receptor-mutant. The other possible scenario is that progestin signaling still functions due to incomplete knockout some of these progestin receptors. Further studies are required to determine like causes of remained oocyte maturation activities in this combined mutant.

To our surprise, Pgrmcs and mPRs are essential for oocyte survival at early female-like gonads. This is the first illustration demonstrating that progestin receptors play a role in zebrafish

sex determination. The underlying mechanism is still unclear. Previous studies showed both Pgrmc1 and mPRs are involved in the anti-apoptosis function of progesterone in granulosa cells (Peluso et al., 2006; Peluso et al., 2008b; Thomas, 2012). Loss of Pgrmcs and mPRs may cause higher apoptosis among early oocytes, leading to the all-male phenotype in combined knockouts of Pgrmc and mPRs (*pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$). The next logical step would be to determinine changes of the gene expression in *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ female-like gonads. Since exogenous estradiol exposure could partially rescue the *pgrmc1*$^{-/-}$*pgrmc2*$^{-/-}$*mprs*$^{-/-}$ females, further experiments are necessary to determine the steroid hormone levels in mutant fish, especially androgens and estrogens. It will be also interesting to investigate the expression of estrogen receptors.

A better understanding of progestin receptor involved functions has great implication for the development of targeted treatments for fertility/infertility. Further research is needed to determine unknown progestin receptor(s), and the interactions of Pgrmcs and mPRs in oocyte survival. Elucidating the mechanisms underlying crosstalks of Pgr, Pgrmcs and mPRs will also advance our knowledge on a wide arrays of progestin signal pathways.

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APPENDIX: IACUC Approval Letter

ECU
Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | 252-744-2355 fax

February 18th, 2020

Yong Zhu, Ph.D.
Department of Biology
East Carolina University

Dear Dr. Blakeslee:

Your Animal Use Protocol entitled, " Studies of Hormones and Receptors in Zebrafish " (AUP #Q185f) was reviewed by this institution's Animal Care and Use Committee on 2/11/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2017

Project Title:

Studies of Hormones and Receptors in Zebrafish

	Principal Investigator	Secondary Contact
Name	Yong Zhu	Fadi Issa
Dept.	Biology	Biology
Office Ph #	252-328-6504	252-328-5446
Cell Ph #	252-227-9695	404-644-1399
Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	Click here to enter text.	Click here to enter text.
Email	zhuy@ecu.edu	ISSAF14@ECU.EDU

For IACUC Use Only

AUP #	Q185f		
New/Renewal	Renewal		
Full Review/Date	DR/Date 2/10/2020		
Approval Date	2/18/2020		
Study Type			
Pain/Distress Category			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC 11/2017	EHS 2/17/2020
OHP Enrollment			
Mandatory Training			
Amendments Approved			

I. Personnel

A. Principal Investigator(s):

Yong Zhu

B. Department(s):

Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Yong Zhu/PhD	Professor/PI/Supervisor	Yes	Have relevant animal experience and training with fish since 1986, know well zebrafish husbandry requirements, spawning protocol, and microinjection procedure etc., responsible for training all collaborators and associates working in the lab
Fadi Issa/PhD	Assistant Professor/co-investigator/co-Supervisor	Yes	Over 10 yrs with fish, helping training associates
Myon He Lee/PhD	Associate Professor/Co-Investigator/co-supervisor	Yes	three year with zebrafish, trained directly by PI (Zhu), helping training associates
Xinjun Wu/MS	PhD student/Student/Research Assistant	Yes	5 years with zebrafish, trained by PI (Zhu)
Jonathan Jacob Carver	Undergraduate student/student/research assistant	Yes	2 years with zebrafish, trained by PI (Zhu)
McKenzie Byrnes	Undergraduate student/student/research assistant	Yes	2 years with zebrafish, trained by PI (Zhu)
Joyel Stephen Puthuparampil	Undergraduate student/student/research assistant	Yes	1 year with zebrafish, trained by PI (Zhu)